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# SOME EFFECTS OF STORAGE CONDITIONS ON CERTAIN DISEASES OF LEMONS [1]

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## INTRODUCTION

The presence of carbon dioxide in a lemon-storage room is quite generally regarded as harmful. Many rooms are operated under instructions that the carbon dioxide in the atmosphere must be kept below 0.5 percent.

Low temperature is known to be harmful to lemons, and exposure to temperatures below 50° F., even during a shipping period of 1 or 2 weeks, is usually carefully avoided.

The present study was undertaken to obtain more definite information in regard to the carbon dioxide and low-temperature tolerance of lemons (*Citrus limonia* Osbeck) and the nature of the injuries resulting from unfavorable storage conditions.

In the course of the experiments several different diseases developed, and in most cases the frequency of their occurrence was modified by the storage treatment.

## MATERIALS AND METHODS

The lemons used in the experiments were forwarded under ventilation in carlot shipments from Los Angeles, Calif.,[2] to New York City and were then shipped by ordinary express to Washington, D. C. They were hauled to the Arlington Experiment Farm, Arlington, Va., and the experiments were started there within a day from the time of arrival in Washington.

The term "green lemons" is used for fruit that at the time of shipment was dark green in color with only very slight traces of yellow, the term "silver lemons" for fruit that was turning from green to yellow, and the term "mature lemons" for fruit that was bright yellow and of proper maturity for immediate marketing. At the time the experimental treatments were begun, the green and silver lemons had slightly too much yellow to be typical for the terms applied.

In the earlier experiments, 20 to 30 lemons of a particular maturity were used under each storage condition. In the later experiments, this number was increased to 50 or 60 lemons for most of the tests. The lemons were selected individually, and great care was taken that the different lots should be alike in maturity, firmness, and general appearance.

It was intended that the relative humidity of the storage rooms should be maintained at about 88 percent; but it sometimes fell

[1] Received for publication Dec. 30, 1936; issued February 1937.

[2] The writers are indebted to C. W. Mann, of the Division of Fruit and Vegetable Crops and Diseases, for the selection and shipment of the fruit.

several points below this for short periods, especially in the 60° F. room. All records of temperature are reported in degrees Fahrenheit.

In part of the carbon dioxide experiments the lemons were held in 5-gallon jars during the period of treatment. In some cases the desired atmosphere was maintained by continuous renewal with air to which carbon dioxide had been added by means of flowmeters. In other cases the carbon dioxide was built up by means of the respiration of the lemons. In the latter tests a fair degree of uniformity was secured by occasional analyses and the regulation of a small opening in the container. In other tests a high percentage of carbon dioxide was maintained for short periods by the use of solid carbon dioxide as a supplement to ordinary ice in pony refrigerators. In these experiments both the carbon dioxide and the temperature were lower at the end of the treatment than at the beginning.

The waxes used in the experiments were prepared by mixing paraffin and mineral oil in approximately equal proportions. In some cases beeswax was substituted for part of the paraffin. There was no indication that the results were affected by modifications in the formula for the wax.

## DECAY

### CHARACTERISTICS

The most common form of decay was that caused by *Alternaria citri* Pierce. Much of this decay was of the "center" type, the central axis or core of the lemon becoming black and soft, with little external evidence of the disease, as is shown in figure 1, *A*. In other cases the entire lemon became black and soft, the decay usually starting at the button and moving toward the stylar end (fig. 1, *B*).

*Penicillium digitatum* Sacc. was a close second to *Alternaria* as a cause of decay, and *P. italicum* Wehmer was of somewhat less common occurrence. The type of decay produced by the last-named is shown in figure 1, *C*.

### EFFECT OF TEMPERATURE

During the first 6 to 8 weeks of storage there was greater development of decay at the higher than at the lower temperatures. This was especially true of alternaria decay. When the storage period was prolonged to 10 weeks or more there was sometimes more decay at 32° and 36° F. than at any of the higher temperatures, especially with fruit that had been stored at the green or the silver stage of maturity. This greater decay at the lower temperatures was largely due to one or the other of the *Penicillium* species and was apparently the result of a prior development of pitting, watery break-down, or other low-temperature trouble that gave the decay organisms favorable points of entrance.

In lemons that were held 1 or 2 weeks at 32°, 36°, or 40° F. before being stored at 50° or 60° there was in general but slightly less decay than in the fruit held continuously at the higher temperature, especially after long periods of storage.

### EFFECT OF CARBON DIOXIDE AND WAXING

It was pointed out in an earlier publication [3] that carbon dioxide has a decidedly inhibiting effect on penicillium decay of oranges and

[3] BROOKS, C., BRATLEY, C. O., and MCCOLLOCH, L. P. TRANSIT AND STORAGE DISEASES OF FRUITS AND VEGETABLES AS AFFECTED BY INITIAL CARBON DIOXIDE TREATMENTS. U. S. Dept. Agr. Tech. Bull. 519, 24 pp., illus. 1936.

FIGURE 1.—Decay of lemons caused by *Alternaria citri* (*A* and *B*) and by *Penicillium italicum* (*C*). Note the spread along the central axis in *A* and the more lightly colored zone around the central area in *C*.

grapefruit. In the present experiments the effect of carbon dioxide was tested on lemons that had been inoculated with *Alternaria citri*. The development of this decay was slow under all conditions, but the progress was apparently as great in an atmosphere containing 40 percent of carbon dioxide as in one that was practically free from carbon dioxide.

A record of decay was kept in all the carbon dioxide and waxing experiments that are reported in detail under pitting and membranous stain. The results gave no indication that either waxing or continuous exposure to low percentages of carbon dioxide (10 percent or less) had any significant effect upon the percentage of either penicillium or alternaria decay at the end of long storage periods.

Lemons that were held in tight containers for long-period carbon dioxide treatments or as controls for these treatments developed more decay in some instances than the fruit that was held in commercial packages at the same temperature. This difference was apparently due to a higher humidity in the tight containers. Fawcett, Klotz, and Nixon [4] have reported experiments in which alternaria decay was reduced by air conditioning.

## PITTING AND PETECA

### CHARACTERISTICS

Pitting, as the name implies, is characterized by the development of pits or depressions in the rind of the fruit,[5] as shown in figure 2, *A* and *B*. In the present experiments it took various forms, its characteristics as well as its prevalence varying with the treatment and apparently with the maturity and vitality of the fruit. In many cases the color of the rind in the pitted area remained normal or nearly so; in other cases it became light brown or a dark brown approaching black.

The pits were usually as much as 0.2 inch in diameter and often more than 0.5 inch, but under certain storage conditions a smaller and shallower type prevailed, giving the lemons a somewhat speckled appearance, as shown in figure 2, *C*. This type was particularly common in 36° storage.

Peteca resembles pitting, but the surface of the peel is relatively slow in losing its color and in the early stages the oil glands are darker than the surrounding tissue.[5] This is shown in figure 2, *D*.

### EFFECT OF TEMPERATURE

The effect of temperature upon the development of pitting and peteca is shown in figures 3 to 9, inclusive. Pitting did not occur in storage at 60° F. It was usually absent and never of importance with either green or mature lemons held at 50°, but in a few instances the silver lemons developed the disease in 50° storage. At 32°, 36°, and 40° the lemons were often badly pitted, especially when held in storage more than 8 to 10 weeks. After the lemons had been 8 weeks or more in storage the disease was much worse at 32° and 36° than at 40°, and with green lemons it was usually worse at 36° than at 32°. These contrasting temperature effects did not disappear when the

[4] FAWCETT, H. S., KLOTZ, L. J., and NIXON, H. W. EFFECTS OF STORAGE AND HOLDING CONDITIONS ON ALTERNARIA IN LEMONS. Calif. Citrogr. 21: 118, 143–144. 1936.

[5] FAWCETT, H. S., and LEE, H. A. CITRUS DISEASES AND THEIR CONTROL. New York. 1926. [Also ed. 2, by Fawcett. 1936.]

fruit was held at room temperature for a week after removal from storage; in fact, they were usually somewhat emphasized by such holding. The pits developed at 40° were often darker than those at 32°. This may have been due to greater aging, greater oxidation, or perhaps to some other cause.

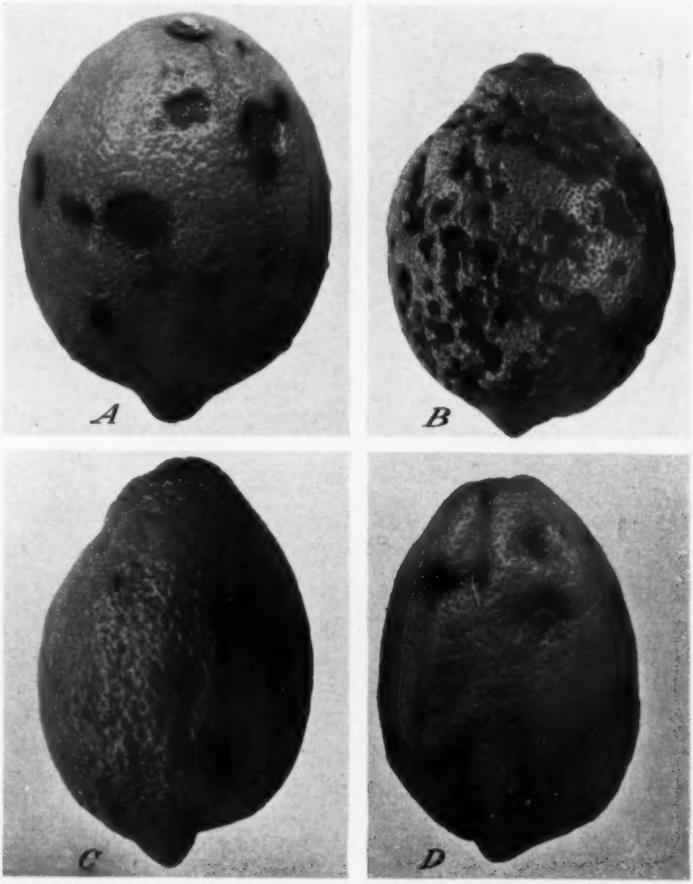


FIGURE 2.—Pitting and peteca: *A*, Lemon showing deep pitting, the usual type in storage at 32° and 40° F.; *B*, pitting on a green lemon that was held at 36° for 13 weeks; *C*, lemon showing shallow pitting, the most common type in storage at 36°, and also one deep pit; *D*, peteca on lemon, as found after shipment from California to Washington, D. C.

Green, silver, and mature lemons that were held at 32°, 36°, or 40° F. for 1 or 2 weeks and were then stored at 60° remained free from pitting. Lemons similarly held at 32° and stored at 50° remained as free from pitting on the average as those held continuously at 50°. Lemons similarly held at 36° or 40° before being stored at 50° showed a slight increase in pitting in some instances, as compared with those held continuously at 50°. There was little to indicate that holding

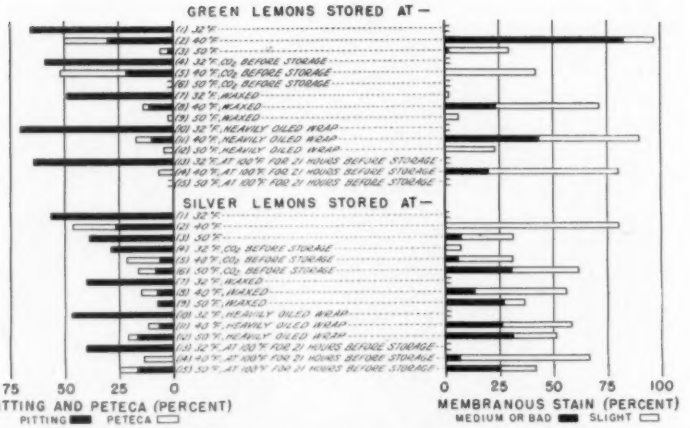
GREEN LEMONS STORED AT —
(1) 32°F.
(2) 40°F.
(3) 50°F.
(4) 32°F. $CO_2$ BEFORE STORAGE
(5) 40°F. $CO_2$ BEFORE STORAGE
(6) 50°F. $CO_2$ BEFORE STORAGE
(7) 32°F. WAXED
(8) 40°F. WAXED
(9) 50°F. WAXED
(10) 32°F. HEAVILY OILED WRAP
(11) 40°F. HEAVILY OILED WRAP
(12) 50°F. HEAVILY OILED WRAP
(13) 32°F. AT 100°F. FOR 21 HOURS BEFORE STORAGE
(14) 40°F. AT 100°F. FOR 21 HOURS BEFORE STORAGE
(15) 50°F. AT 100°F. FOR 21 HOURS BEFORE STORAGE
SILVER LEMONS STORED AT —
(1) 32°F.
(2) 40°F.
(3) 50°F.
(4) 32°F. $CO_2$ BEFORE STORAGE
(5) 40°F. $CO_2$ BEFORE STORAGE
(6) 50°F. $CO_2$ BEFORE STORAGE
(7) 32°F. WAXED
(8) 40°F. WAXED
(9) 50°F. WAXED
(10) 32°F. HEAVILY OILED WRAP
(11) 40°F. HEAVILY OILED WRAP
(12) 50°F. HEAVILY OILED WRAP
(13) 32°F. AT 100°F. FOR 21 HOURS BEFORE STORAGE
(14) 40°F. AT 100°F. FOR 21 HOURS BEFORE STORAGE
(15) 50°F. AT 100°F. FOR 21 HOURS BEFORE STORAGE
75 50 25 0
PITTING AND PETECA (PERCENT)
PITTING
PETECA
0 25 50 75 100
MEMBRANOUS STAIN (PERCENT)
MEDIUM OR BAD
SLIGHT

FIGURE 3.—Effect of storage conditions on pitting, peteca, and membranous stain. Green and silver lemons stored at various temperatures November 28, 1933; record made 14 weeks later. In the lots receiving $CO_2$ before storage, the fruit was held for 64 hours in an atmosphere in which the carbon dioxide dropped from 50 percent to 30 percent and averaged 36 percent and in which the temperature dropped from 70° to 50° F. and averaged 56°.

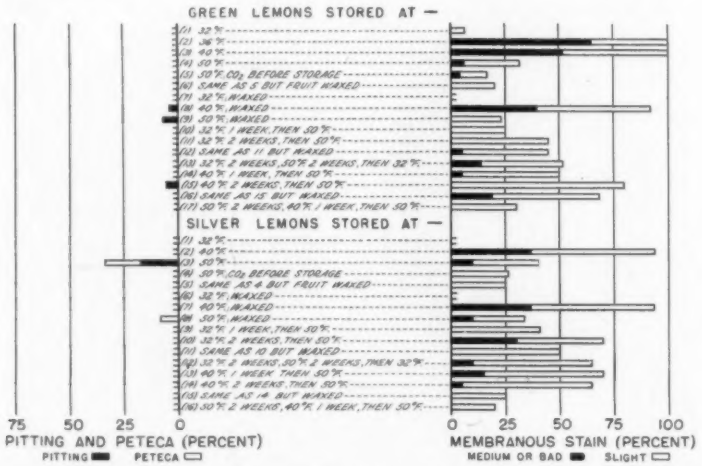
GREEN LEMONS STORED AT —
(1) 32°F.
(2) 36°F.
(3) 40°F.
(4) 50°F.
(5) 50°F. $CO_2$ BEFORE STORAGE
(6) SAME AS 5 BUT FRUIT WAXED
(7) 32°F. WAXED
(8) 40°F. WAXED
(9) 50°F. WAXED
(10) 32°F. 1 WEEK, THEN 50°F.
(11) 32°F. 2 WEEKS, THEN 50°F.
(12) SAME AS 11 BUT WAXED
(13) 32°F. 2 WEEKS, 50°F. 2 WEEKS, THEN 32°F.
(14) 40°F. 1 WEEK, THEN 50°F.
(15) 40°F. 2 WEEKS, THEN 50°F.
(16) SAME AS 15 BUT WAXED
(17) 50°F. 2 WEEKS, 40°F. 1 WEEK, THEN 50°F.
SILVER LEMONS STORED AT —
(1) 32°F.
(2) 40°F.
(3) 50°F.
(4) 50°F. $CO_2$ BEFORE STORAGE
(5) SAME AS 4 BUT FRUIT WAXED
(6) 32°F. WAXED
(7) 40°F. WAXED
(8) 50°F. WAXED
(9) 32°F. 1 WEEK, THEN 50°F.
(10) 32°F. 2 WEEKS, THEN 50°F.
(11) SAME AS 10 BUT WAXED
(12) 32°F. 2 WEEKS, 50°F. 2 WEEKS, THEN 32°F.
(13) 40°F. 1 WEEK, THEN 50°F.
(14) 40°F. 2 WEEKS, THEN 50°F.
(15) SAME AS 14 BUT WAXED
(16) 50°F. 2 WEEKS, 40°F. 1 WEEK, THEN 50°F.
75 50 25 0
PITTING AND PETECA (PERCENT)
PITTING
PETECA
0 25 50 75 100
MEMBRANOUS STAIN (PERCENT)
MEDIUM OR BAD
SLIGHT

FIGURE 4.—Effect of storage conditions on pitting, peteca, and membranous stain. Green and silver lemons stored at various temperatures March 20, 1934; record made 12 weeks later. In the lots receiving $CO_2$ before storage the fruit was held for 48 hours at 59° F. in an atmosphere in which the carbon dioxide was maintained at 50 percent by continuous air renewal.

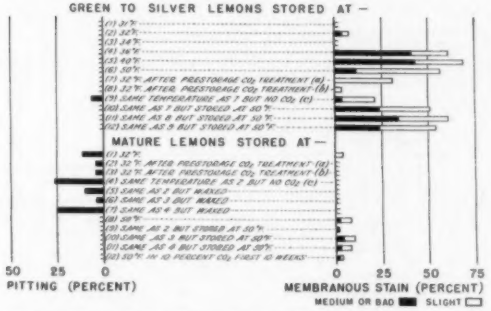


FIGURE 5.—Effect of storage conditions on pitting and membranous stain (no peteca developed) of lemons stored at various temperatures June 23, 1934. Part of the lemons were about halfway between the green and silver stages in maturity and the others were fully mature; record made after 12 weeks' storage: *a*, Lemons in a pony refrigerator with the temperature dropping from 73° to 49° F. in 42 hours and averaging 51°, and the carbon dioxide dropping from 59 percent to 30 percent and averaging 40 percent; *b*, temperature as above, but the carbon dioxide dropping from 33 percent to 14 percent and averaging 22 percent; *c*, controls, with no carbon dioxide.

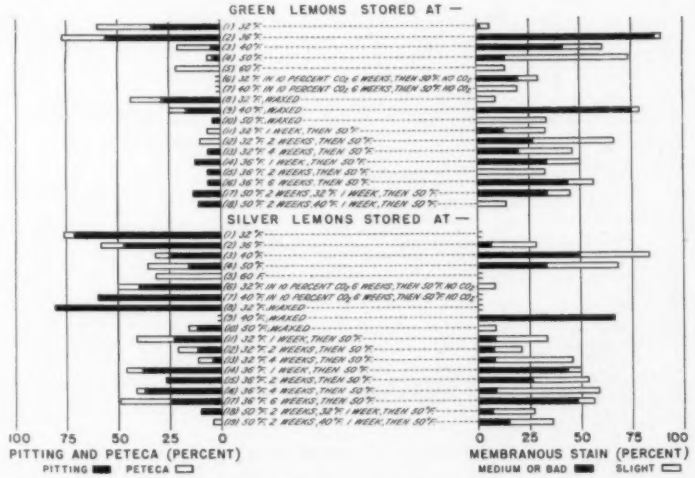


FIGURE 6.—Effect of storage conditions on pitting, peteca, and membranous stain. Green and silver lemons stored at various temperatures January 23, 1935; record made 16 weeks later. In the lots exposed to $CO_2$ the gas was built up by the respiration of the lemons and the treatments were continued during the first 6 weeks of storage.

the fruit at a lower temperature for 1 or 2 weeks, or even 4 weeks, produced any decided tendency to pitting.

In the experiments reported in figure 3, fruit that was held at 100° F. for 21 hours before being stored had slightly less pitting than the control fruit, but the difference could hardly be regarded as significant.

There seemed to be little evidence that the development of peteca was modified by temperature conditions. Its erratic occurrence with some lots of fruit seemed to indicate that its prevalence was largely determined by prestorage conditions.

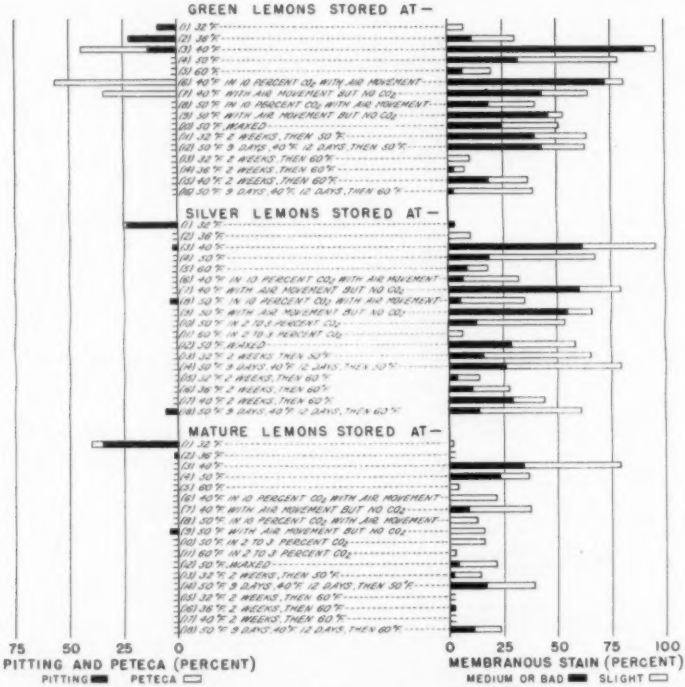


FIGURE 7.—Effect of storage conditions on pitting, peteca, and membranous stain of lemons stored at various temperatures June 26, 1935. The carbon dioxide treatments were continued during the first 3 weeks of storage. The record on the silver and mature lemons was after 9 weeks' storage, that on the green lemons after 14 weeks' storage.

## EFFECT OF CARBON DIOXIDE

In several of the experiments lemons were exposed to high percentages of carbon dioxide for a period of 2 or 3 days before being placed in storage. The results are shown in figures 3 to 5, inclusive. In four tests with green lemons, four with silver lemons, and two with mature lemons, pitting was decreased by this prestorage gas treatment, and there was no instance in which it was increased. The decreases were slight in some cases, but in others they ran as high as 20, 40, and 60 percent.

In other experiments the lemons were held for several weeks in atmospheres containing 2 or 10 percent of carbon dioxide. In some

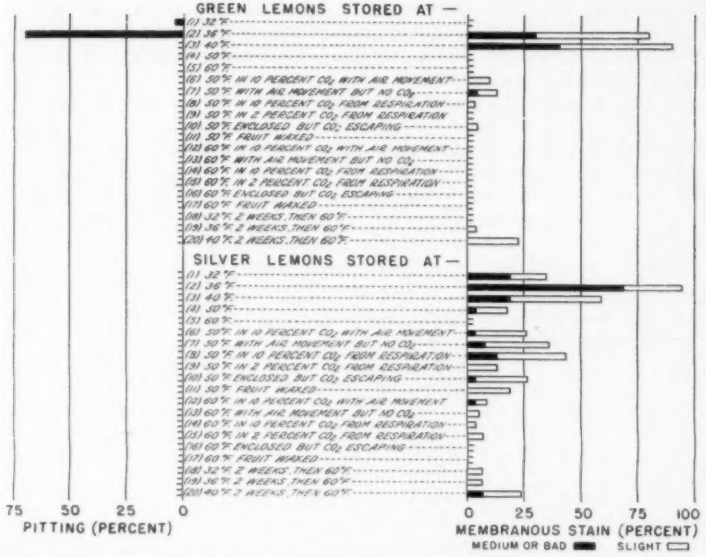
GREEN LEMONS STORED AT —
(1) 32°F.
(2) 36°F.
(3) 40°F.
(4) 50°F.
(5) 60°F.
(6) 50°F. IN 10 PERCENT $CO_2$ WITH AIR MOVEMENT
(7) 50°F. WITH AIR MOVEMENT BUT NO $CO_2$
(8) 50°F. IN 10 PERCENT $CO_2$ FROM RESPIRATION
(9) 50°F. IN 2 PERCENT $CO_2$ FROM RESPIRATION
(10) 50°F. ENCLOSED BUT $CO_2$ ESCAPING
(11) 50°F. FRUIT WAXED
(12) 60°F. IN 10 PERCENT $CO_2$ WITH AIR MOVEMENT
(13) 60°F. WITH AIR MOVEMENT BUT NO $CO_2$
(14) 60°F. IN 10 PERCENT $CO_2$ FROM RESPIRATION
(15) 60°F. IN 2 PERCENT $CO_2$ FROM RESPIRATION
(16) 60°F. ENCLOSED BUT $CO_2$ ESCAPING
(17) 60°F. FRUIT WAXED
(18) 32°F. 2 WEEKS, THEN 60°F.
(19) 36°F. 2 WEEKS, THEN 60°F.
(20) 40°F. 2 WEEKS, THEN 60°F.
SILVER LEMONS STORED AT —
(1) 32°F.
(2) 36°F.
(3) 40°F.
(4) 50°F.
(5) 60°F.
(6) 50°F. IN 10 PERCENT $CO_2$ WITH AIR MOVEMENT
(7) 50°F. WITH AIR MOVEMENT BUT NO $CO_2$
(8) 50°F. IN 10 PERCENT $CO_2$ FROM RESPIRATION
(9) 50°F. IN 2 PERCENT $CO_2$ FROM RESPIRATION
(10) 50°F. ENCLOSED BUT $CO_2$ ESCAPING
(11) 50°F. FRUIT WAXED
(12) 60°F. IN 10 PERCENT $CO_2$ WITH AIR MOVEMENT
(13) 60°F. WITH AIR MOVEMENT BUT NO $CO_2$
(14) 60°F. IN 10 PERCENT $CO_2$ FROM RESPIRATION
(15) 60°F. IN 2 PERCENT $CO_2$ FROM RESPIRATION
(16) 60°F. ENCLOSED BUT $CO_2$ ESCAPING
(17) 60°F. FRUIT WAXED
(18) 32°F. 2 WEEKS, THEN 60°F.
(19) 36°F. 2 WEEKS, THEN 60°F.
(20) 40°F. 2 WEEKS, THEN 60°F.
75 50 25 0
PITTING (PERCENT)
0 25 50 75 100
MEMBRANOUS STAIN (PERCENT)
MEDIUM OR BAD
SLIGHT

FIGURE 8.—Effect of storage conditions on pitting and membranous stain of lemons stored at various temperatures February 17, 1936; record made 16 weeks later. The carbon dioxide treatments were continued during the first 4 weeks of storage.

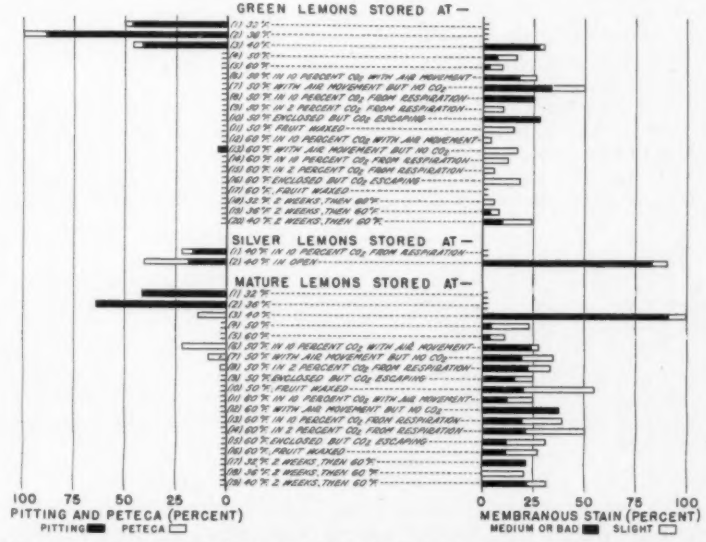
GREEN LEMONS STORED AT —
(1) 32°F.
(2) 36°F.
(3) 40°F.
(4) 50°F.
(5) 60°F.
(6) 50°F. IN 10 PERCENT $CO_2$ WITH AIR MOVEMENT
(7) 50°F. WITH AIR MOVEMENT BUT NO $CO_2$
(8) 50°F. IN 10 PERCENT $CO_2$ FROM RESPIRATION
(9) 50°F. IN 2 PERCENT $CO_2$ FROM RESPIRATION
(10) 50°F. ENCLOSED BUT $CO_2$ ESCAPING
(11) 50°F. FRUIT WAXED
(12) 60°F. IN 10 PERCENT $CO_2$ WITH AIR MOVEMENT
(13) 60°F. WITH AIR MOVEMENT BUT NO $CO_2$
(14) 60°F. IN 10 PERCENT $CO_2$ FROM RESPIRATION
(15) 60°F. IN 2 PERCENT $CO_2$ FROM RESPIRATION
(16) 60°F. ENCLOSED BUT $CO_2$ ESCAPING
(17) 60°F. FRUIT WAXED
(18) 32°F. 2 WEEKS, THEN 60°F.
(19) 36°F. 2 WEEKS, THEN 60°F.
(20) 40°F. 2 WEEKS, THEN 60°F.
SILVER LEMONS STORED AT —
(1) 40°F. IN 10 PERCENT $CO_2$ FROM RESPIRATION
(2) 40°F. IN OPEN
MATURE LEMONS STORED AT —
(1) 32°F.
(2) 36°F.
(3) 40°F.
(4) 50°F.
(5) 60°F.
(6) 50°F. IN 10 PERCENT $CO_2$ WITH AIR MOVEMENT
(7) 50°F. WITH AIR MOVEMENT BUT NO $CO_2$
(8) 50°F. IN 2 PERCENT $CO_2$ FROM RESPIRATION
(9) 50°F. ENCLOSED BUT $CO_2$ ESCAPING
(10) 50°F. FRUIT WAXED
(11) 60°F. IN 10 PERCENT $CO_2$ WITH AIR MOVEMENT
(12) 60°F. WITH AIR MOVEMENT BUT NO $CO_2$
(13) 60°F. IN 10 PERCENT $CO_2$ FROM RESPIRATION
(14) 60°F. IN 2 PERCENT $CO_2$ FROM RESPIRATION
(15) 60°F. ENCLOSED BUT $CO_2$ ESCAPING
(16) 60°F. FRUIT WAXED
(17) 32°F. 2 WEEKS, THEN 60°F.
(18) 36°F. 2 WEEKS, THEN 60°F.
(19) 40°F. 2 WEEKS, THEN 60°F.
100 75 50 25 0
PITTING AND PETECA (PERCENT)
PITTING
PETECA
0 25 50 75 100
MEMBRANOUS STAIN (PERCENT)
MEDIUM OR BAD
SLIGHT

FIGURE 9.—Effect of storage conditions on pitting, peteca, and membranous stain of lemons stored at various temperatures May 12, 1936; record made 14 weeks later. The carbon dioxide treatments were continued during the first 4 weeks of storage.

of these tests the carbon dioxide was built up by the respiration of the fruit, and in others it was obtained by continuous renewal with an atmosphere to which carbon dioxide had been added. The results are shown in figures 5 to 9, inclusive.

In the experiment reported in figure 5, lemons were held at 50° F. in 10 percent of carbon dioxide for 10 weeks without the development of pitting, but neither was there pitting in the control lot in the open.

In the experiments reported in figure 6, the fruit was held at 32° and at 40° F. during 6 weeks' exposure to an atmosphere containing 10 percent of carbon dioxide. No control lot was held at these temperatures for the same length of time as the treated fruit, and it is therefore impossible to draw definite conclusions. There is some indication that the carbon-dioxide treatments caused a decided decrease in the pitting of green lemons but possibly a slight increase in the pitting of silver lemons.

The experiments reported in figure 7 were carried out at 40° and 50° F., and those reported in figures 8 and 9 at 50° and 60°. In all cases control lots were held in similar confinement but in atmospheres that were free from any accumulation of carbon dioxide. In the experiments of figure 7 the carbon-dioxide treatments were continued for 3 weeks and in the experiments of figures 8 and 9 for 4 weeks.

In a total of 24 experiments in which the fruit was exposed to 10 percent of carbon dioxide and in 10 experiments in which it was exposed to approximately 2 percent, there were 30 instances in which no pitting developed on either treated or control fruit. In the experiments in which the fruit was exposed to 10 percent of carbon dioxide there were three instances in which there was more pitting on the control fruit than on the treated lot and but one instance in which the reverse condition held. The results do not give any indication that exposure to low percentages of carbon dioxide had any tendency to cause pitting.

In the 34 carbon-dioxide tests mentioned above there were 4 instances in which peteca occurred and in 3 of these it was worse on the treated than on the control fruit. If peteca is to be considered a form of pitting, this record just balances the favorable contrast reported above, leaving the effect of low percentages of carbon dioxide null.

### EFFECT OF OILED WRAPPERS AND WAXING

Lemons that were waxed and those that were packed in heavily oiled wrappers developed less pitting than those stored without these treatments. This is shown in figures 3 to 9, inclusive. When compared with the controls, the treated fruit showed approximately three times as many instances of a definite decrease as of a definite increase in pitting. The results indicate a similar or even greater decrease in the peteca form of pitting on the fruit that received an oiling or waxing treatment.

## RED BLOTCH

Red blotch, or adustiosis, is a peel defect that is much shallower and more diffuse than pitting, although in some instances it seems to shade gradually into the milder or shallower type of pitting (p. 798). The diseased area is reddish brown in color, and in early and marginal stages of the disease it appears to be made up of an aggregation of small brown dots, but later a more continuous browning develops.

In the present experiments red blotch has been of relatively rare and rather erratic occurrence. It was not found on lemons that were mature when placed in storage and was far more common on green than on silver lemons. It was found almost exclusively on fruit stored at 36° or 40° F., with an occasional occurrence at 32° and 50° but never at 60°.

Either by chance or otherwise, the lots that were exposed to carbon dioxide remained entirely free of the disease. Fruit that was waxed or held in heavily oiled wrappers developed as much red blotch as the controls.

## MEMBRANOUS STAIN

### CHARACTERISTICS

Membranous stain, or membranosis, is characterized by a browning or darkening of the membranes or carpellary walls between the segments. This condition is shown in figure 10. The central core tissues and the inner tissues of the rind are also sometimes affected. The disease can be detected only when the lemons are cut.

Membranous stain, especially in its milder forms, was by far the most prevalent disease found in the present experiments. The record of its occurrence is shown in figures 3 to 9, inclusive.

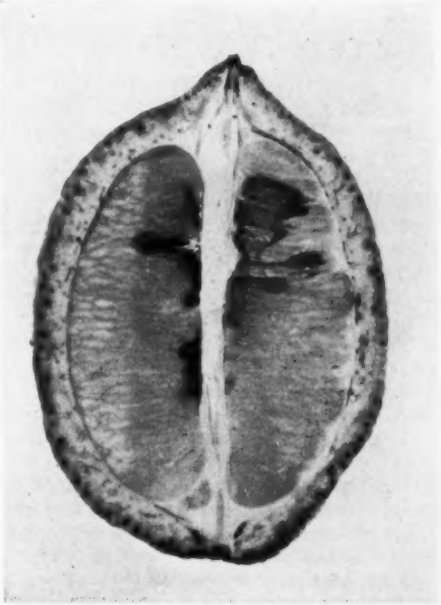

FIGURE 10.—Membranous stain as seen in longitudinal section of a silver lemon that was held 13 weeks at 40° F.

### EFFECT OF TEMPERATURE

Membranous stain showed an extreme response to temperature. Lemons stored at 32° F. usually remained practically free from the disease; whereas those held at 40° usually were seriously affected, in many cases but little of the fruit remaining free. In the few tests in which the fruit was held at 31° or 34° the results were similar to those at 32°. There was much less membranous stain at both 36° and 50° than at 40° and far less at 60° than at 36° or 50°, yet the amount of stain at 60° was much greater than at 32°.

In most of the experiments the lemons were held at 70° F. for a week after removal from storage. Membranous stain did not increase during this period. This fact would seem to prove that the elimination of the disease at 32° was permanent and not due to any temporary inhibition of oxidation or related processes.

The extreme temperature response of membranous stain and its sharp divergence from that of pitting make an interesting background for speculation as to the fundamental causes of the two diseases.

With fruit that was moved from one temperature to another the development of membranous stain seemed in general to be in accord with the relative periods at the two temperatures. Fruit held at 32° F. for 1 or 2 weeks before being stored at 50° or 60° usually developed less membranous stain than that held continuously at the higher temperatures, and fruit held at 40° for 1 or 2 weeks before being stored at 50° or 60° usually had more stain than that held continuously at the higher temperatures.

In the experiments reported in figure 3, green and silver lemons that were held at 100° F. for 21 hours before being stored at 40° developed less membranous stain than those that were placed immediately at 40°.

### EFFECT OF CARBON DIOXIDE

Increasing the carbon dioxide content of the storage atmosphere decreased the occurrence of membranous stain.

In the experiments in which the lemons were exposed to a high percentage of carbon dioxide for 2 or 3 days before being stored (figs. 3–5) there were 10 instances in which there was more membranous stain in the controls than in the treated fruit and 5 instances in which the reverse was true. In two lots stored at 32° F. the treated lemons had more of the disease than the controls. At this temperature both the treated and control lots remained remarkably free from membranous stain.

With fruit held for several weeks in constantly renewed atmospheres to which 10 percent of carbon dioxide had been added (figs. 7–9) there were 12 instances in which membranous stain was greater on the control fruit and 2 instances in which it was greater on the treated fruit.

Experiments were made in which the carbon dioxide from respiration was allowed to accumulate in the storage chamber. In 11 of these experiments the carbon dioxide was held at approximately 10 percent and in 12 it was held at 2 to 3 percent (figs. 6–9). There were more instances in which the membranous stain in the control fruit exceeded that in the treated fruit than there were of the reverse condition.

The results indicate that the presence of carbon dioxide in the storage atmosphere is beneficial rather than harmful so far as membranous stain is concerned. The favorable effect of exposure to carbon dioxide, together with the favorable effect of low temperature, would suggest the possibility that the control in both cases is due to suppression of oxidation.

The lack of harmful effects from exposure to carbon dioxide does not prove that some form of ventilation or air circulation may not be beneficial. There may be other respiratory products that accumulate to a harmful degree. A comparison of the fruit that was confined, either for carbon dioxide treatment or as controls, with that held in the open (figs. 5–9) shows that there were a few more instances of membranous stain on the confined fruit exceeding that on the fruit in the open than there were of the reverse condition; but the contrast is hardly great enough to suggest a practical significance.

### EFFECT OF OILED WRAPPERS AND WAXING

Waxing the lemons tended to decrease the development of membranous stain (figs. 3, 4, 6–9). At 32° and 60° F. little membranosis developed on either treated or control lots. When compared with the controls, the treated fruit showed approximately four times as many instances of a distinct decrease as of a distinct increase in

membranous stain at 40° and 50°. The few experiments with heavily oiled wrappers gave results that were favorable rather than otherwise.

Waxing and the use of heavily oiled wrappers would be expected to increase the carbon dioxide and decrease the oxygen content of the internal atmosphere of the fruit, and it is possible that their favorable effect is to be explained on the same basis as that of the carbon dioxide treatments.

## WATERY BREAK-DOWN

### CHARACTERISTICS

In low-temperature storage the lemons sometimes became water-soaked, soft, and spongy, as if frozen. Both the peel and the pulp were usually involved. The term "watery break-down" is suggested for this diseased condition. A similar disease has been reported in grapefruit.[6]

### STORAGE RESPONSE

The disease was confined to lemons held for long periods in storage at 31° or 32° F. In some cases 25 percent or more of the fruit was affected at the time of removal and there was usually an equal or higher percentage of the fruit that became watery when held for a few days after removal. Green lemons remained relatively free of the disease, but silver and mature lemons became badly affected. Lemons that were exposed to high temperature or to high percentages of carbon dioxide before being stored at 32° had far more of the disease than those that did not receive these treatments.

Watery break-down is evidently a low-temperature suffocation type of disease.

## SCALD

The term "scald" is sometimes used for injuries resulting from chemical treatments or exposure to high temperature. In the present experiments scaldlike injuries were occasionally found in low-temperature storage. With green lemons, the disease was characterized by a general disappearance of the green color and a slight depression of the affected peel. With silver and mature lemons no depression was evident, and the diseased condition was indicated by a color change of the peel from normal yellow to a honey yellow or cinnamon buff.[7] The trouble was sometimes associated with watery break-down and is probably closely related to that disease, yet no instance was observed in which one disease developed into the other.

## INTERNAL DECLINE

Internal decline, or endoxerosis, was found occasionally in the present experiments, but with little if any reference to storage conditions. The disease is characterized by a breaking down and drying of the internal tissue, particularly that near the stylar end.

## FIRMNESS AND COLOR

Notes were taken on the effects of the different treatments upon the color and firmness of the fruit, and the results were recorded as percentages. Lemons showing ideal firmness were given a rating of 100 and softer lemons a lower rating. Fruit that had the most desirable color for immediate marketing was rated at 100; that which was

[6] BROOKS, C., and MCCOLLOCH, L. P. SOME STORAGE DISEASES OF GRAPEFRUIT. Jour. Agr. Research 52: 319-351, illus. 1936.

[7] RIDGWAY, R. COLOR STANDARDS AND COLOR NOMENCLATURE. 43 pp., illus. Washington, D. C. 1912.

greener was given a lower rating and that which had too much brown in the yellow was given a higher rating. The results from two of the experiments are shown in figures 11 and 12. The results from the other experiments were in general agreement with these but showed slightly less contrast.

FIGURE 11.—Effect of storage conditions upon the firmness and color of lemons. Green and silver lemons stored March 20, 1934; record made 12 weeks later.

The effects upon color were more pronounced with green lemons than with silver lemons. The rate of color development usually increased with an increase in the storage temperature.

FIGURE 12.—Effect of storage conditions upon the firmness and color of lemons. Green and silver lemons stored January 23, 1935; record made 16 weeks later.

Carbon dioxide treatments usually delayed color development, especially in the prestorage treatments with high percentages of carbon dioxide.

Color usually developed more slowly in waxed than in unwaxed fruit.

The firmness of the fruit usually decreased with a rise in the storage temperature. Fruit held for 1 to 3 weeks at a lower temperature before being moved to a higher usually showed decidedly greater firmness than that held continuously at the higher temperature.

Carbon dioxide treatments had little, if any, effect upon loss of firmness.

Waxing had a most pronounced effect in delaying the loss in firmness, especially at the higher temperatures.

In the tests reported in figure 11 several of the waxed and unwaxed lots were weighed before and after being stored, and it was found that the loss in weight of the waxed fruit was 75 to 95 percent less than that of the control fruit.

## SUMMARY

Certain storage diseases of lemons are described and experiments are reported showing the response of these diseases to storage conditions.

The storage lots were small but great care was taken in selecting them.

Fruit was held in storage at 32°, 36°, 40°, 50°, and 60° F.

During the first 6 or 8 weeks of storage there was more decay at the higher temperatures, but with longer storage this was sometimes reversed, apparently because of decay following watery break-down and pitting.

Carbon dioxide failed to check alternaria decay.

Pitting did not occur in 60° F. storage and was seldom serious at 50°, but was the great limiting factor in storage at lower temperatures. It was much worse at 32° and 36° than at 40°.

Holding the lemons at 32°, 36°, or 40° F. for 1 or 2 weeks before storing them at a higher temperature did not increase pitting.

Prestorage treatments with high percentages of carbon dioxide tended to decrease pitting.

Waxed lemons developed less pitting than unwaxed ones.

Membranous stain showed most pronounced temperature response. Its occurrence at 32°, 36°, 40°, 50°, and 60° F. stood approximately in the order of 1, 12, 22, 8, and 2 percent respectively, with usually only a small amount of the disease at 32° and 75 to 100 percent of the fruit affected at 40°.

Fruit held at 32° F. 1 or 2 weeks before being stored at 50° or 60° usually developed less membranous stain than that held continuously at the higher temperature, but when held at 40° 1 or 2 weeks before being stored at 50° or 60° it sometimes developed more.

Carbon dioxide gas storage decreased membranous stain. This was true with fruit receiving prestorage treatments with high percentages of carbon dioxide and with that exposed for several weeks to low percentages produced either by the addition of carbon dioxide to the atmosphere or by building it up by means of the fruit respiration.

Waxed fruit usually had less membranous stain than unwaxed.

Watery break-down was sometimes serious in 32° F. storage but did not occur at higher temperatures. Scald and red blotch were occasionally found on fruit held at low temperatures.

Prestorage treatments with high percentages of carbon dioxide tended to delay color development.

Waxed fruit lost weight and firmness very much more slowly than unwaxed fruit.

No injury resulted from the accumulation of low percentages of carbon dioxide in the storage atmosphere, and none from prestorage exposure to high percentages of carbon dioxide.

# PRODUCTION OF SYNTHETIC MYCORHIZA IN THE CULTIVATED CRANBERRY[1]

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## INTRODUCTION

Investigations in the Ericaceae have played an important role in the broader study of relationships existing between mycorhizal fungi and their host plants. Many species of the family, particularly those growing in peat and raw humus soils, regularly have mycorhiza of a rather uniform type, in which the epidermal or cortical cells of the roots are filled with compact masses of fungus mycelium. According to some investigators, the mycorhizal fungi invade above-ground organs as well as roots of the host, the mycelium in this case being generally distributed throughout the plant in a highly attenuated and sparsely developed form. Extraordinary significance has been ascribed to this systemic form of infection, as the fungus is considered to furnish an indispensable stimulus to root production by the host plant. Other investigators have questioned the existence of systemic infection and its obligate relation to root production. Some workers suspect that the mycorhizal fungus supplies nitrogen to its host, either by making nitrogenous compounds in the peat available or by directly fixing atmospheric nitrogen; others doubt whether the host derives any benefit from its endophyte. The present paper adds to this general subject some observations on the mycorhiza of the cultivated cranberry, *Vaccinium macrocarpon* Ait.

## REVIEW OF LITERATURE

A critical review of the literature is essential to an understanding of the discordant views held by present investigators. Stahl in 1900 (*32*)[2] grew seedlings of *Vaccinium myrtillus* in sterilized soil and reported that they were mycorhiza-free but developed as well as mycorhiza-infected plants. Ternetz in 1907 (*37*) reported that several strains of a fungus described as *Phoma radicis*, isolated from roots of different ericaceous species, were able to fix atmospheric nitrogen. The identity of the isolated strains with the root endophytes was not established because mycorhiza-free plants could not be secured for inoculation tests, even by growing plants from surface-sterilized seed planted in peat sterilized at 120° C. on 2 successive days. Ternetz reasoned that the mycorhizal fungus must have been present in the seed, and in confirmation reported finding brown hyphae, similar to those occurring on ericaceous roots, in seed coats of *Calluna vulgaris*, one of the plants with which she worked. After considering various ways in which the mycelium might have reached the seed, Ternetz inclined toward the view that infection takes place during bloom.

[1] Received for publication Feb. 3, 1937; issued February 1937.

[2] Reference is made by number (italic) to Literature Cited, p. 834.

In a series of publications beginning in 1911, Rayner gradually developed the hypothesis of systemic infection and obligate symbiosis in the Ericaceae. Her first (joint) paper (*23*) suggested that mycorrhiza might be an important factor in accounting for the natural distribution of *Calluna vulgaris*. Working with the same plant, she reported in 1913 (*16*) finding seed-coat infection in unopened capsules and isolated a fungus from surface-sterilized mature seed which "has since then been identified with the species occurring as mycorhiza in the roots" (*16, p. 70, footnote*). Fungus-free cultures of seedlings were obtained when the strength of the sterilizing solution (mercuric chloride) was increased to 1 percent. Rayner interpreted this to mean that the seed-coat-inhabiting mycorhizal fungus had been effectively eliminated and that in consequence these seedlings were truly mycorhiza-free. All such seedlings failed to produce normal roots. In 1915 (*17*) she reported success in stimulating similar "mycorrhiza-free" seedlings to form roots and grow normally on an agar substrate by inoculating the cultures with a *Phoma* isolated from unopened fruits. The circumstance of root stimulation was considered to prove not only that the *Phoma* was identical with the *Calluna* endophyte but also that the relation between root formation and mycorhizal infection was obligate. As will be pointed out later, the *Phoma* did not produce typical mycorhiza in the inoculated seedlings. Unlike Ternetz, Rayner was of the opinion that the fungus reached the seed by growing up through stem and fruit tissues and that seedlings subsequently became infected by hyphae from seed coats growing into roots shortly after germination. Microscopic examination revealed fine hyphae in leaves and stems of the plant. The final link in the development of Rayner's hypothesis appeared in 1922 (*19*), when she referred her *Phoma* to the nitrogen-fixing *P. radicis* of Ternetz and, by accepting the identity of the fungi, considered that her findings confirmed and extended those of Ternetz.

Christoph in 1921 (*6*) first seriously questioned Rayner's work. He isolated a nonfruiting fungus from roots of *Calluna vulgaris*, which produced typical mycorhiza when inoculated into the soil in which *Calluna* seedlings and cuttings were growing. The fungus was obviously not *Phoma radicis*. Uninoculated plants grew normally and remained free from mycorhiza, in some cases for more than 2 years. Christoph started his plants in sterilized soil, but afterward grew them in open pots unprotected from contamination. Rayner (*18*) promptly replied to this article by criticizing Christoph's work on the three grounds that the seeds were incompletely sterilized, that they were not grown under aseptic conditions, and that the microscopic technique employed in making the examination for infection was inadequate. She charged that Christoph completely overlooked the "fine mycelium" type of infection, which was undoubtedly present in his uninoculated plants. On her part, Rayner minimized the fact that Christoph's fungus produced the "knot" form of mycorhiza in roots of plants growing in previously sterilized soil.

Dufrénoy (*8*) in 1917 reported that the endotrophic mycorhizal fungus associated with root tubercles of *Arbutus unedo* extended throughout all organs of the plant in that species. After death of sepals, petals, and leaves, the fungus continued to grow saprophytically and in that stage produced conidia. The fungus was also said to sporulate on rootlets which it had previously parasitized. By

implication the fungus was considered to be a strain of *Phoma radicis*. It was not isolated from the plant. Working with the same host species, Rivett in 1924 (*26*) concluded that the tubercles were primarily caused by a fungus growing ectotrophically and that the "knot" form of endotrophic mycorhiza developed in them somewhat later. Nontuberculate roots were frequently infected with the attenuated form of hyphae. It was assumed that the three types of mycorhiza—the ectotrophic and both endotrophic forms—were caused by a single fungus, although no isolations were made.

A reinvestigation of the whole problem of mycorhiza in *Calluna* was reported by Rayner (*20*) in 1925. Essentially the same conclusions were reached. Some new details regarding distribution and time of development of mycelium in the plant were given. She stated that "fungal infection and the stimulus to development associated with it on the one hand, and the formation of root mycorhiza on the other hand, must be regarded as distinct phenomena" (*20, p. 285*), although the same fungus was believed to bring about both conditions. When Rayner repeated Christoph's experiment of growing cuttings in sterilized peat, she found that after 8 months the roots appeared to be free from mycelium; i. e., like Christoph's check plants, they failed to develop the "knot" type of mycorhiza. However, she insisted that the attenuated form of the endophyte was present.

In 1928 Doak (*7*) briefly reported isolating a mycorhizal fungus from roots of *Vaccinium corymbosum* and *V. pennsylvanicum*, which produced mycorhiza in "sterile seedlings." Normal root and stem development did not depend on the presence of the fungus. The fungus resembled *Rhizoctonia*. Unfortunately, a complete account of these investigations has not been published.

Using a culture of *Phoma radicis callunae*, isolated by Rayner in 1924, Jones and Smith (*12*) in 1928 reported that the fungus fixed a small amount of atmospheric nitrogen but exhibited unmistakable growth response when available nitrogen was added to the culture solution. The authors felt that the study ought to be repeated with a newly isolated strain of the fungus.

The notion of systemic infection was carried still further by Rayner in 1929 (*21*), when she reported that in the genus *Vaccinium* the endophyte penetrates into the endosperm of the developing seed. The investigation dealt largely with *V. macrocarpon* and *V. oxycoccus*, though ovarial infection by fine hyphae was reported for seven species of the genus. Fruit infection was detected as early as July. In mature seed the embryo alone lacked infection. When seed were aseptically transferred to agar from surface-sterilized berries, the fine hyphae in the seed coat and endosperm became active and penetrated into the developing hypocotyl but did not grow into the agar, a circumstance that appeared "very puzzling" (*21, pp. 60–61*). Despite the fact that the endophyte was never isolated from any part of the cranberry plant, it was assumed that the mycorhizal fungus was a strain of *Phoma radicis*. Seedlings were grown on agar with and without seed coats removed. In both cases they grew well but rooted irregularly, and roots in contact with the agar browned early. Roots and stems of the seedlings were found to be sparsely infected with fine hyphae. It was argued that since the endosperm is infected the seedlings likewise must be infected; consequently, it is impossible to

follow the classic method of inoculating fungus-free plants with a pure culture of the endophyte in *Vaccinium* species. Concerning the failure of the seedlings to develop the "knot" type of mycorhiza in agar cultures, the following statements appear (*21, pp. 66–68*):

> [Mycorrhiza formation] is an annual event, influenced largely by soil conditions. * * * The direct relation of mycorrhiza formation to soil constitution is emphasized once more, and in *Vaccinium* there can be little doubt of its immediate connexion with an abundant supply of natural humus. * * * Mycorrhiza formation [in *Vaccinium*] is likewise hindered by certain conditions in the rooting medium. * * * the formation of mycorrhiza is partially inhibited in the roots of both species [*Vaccinium* and *Calluna*] when grown in a sterilized medium.

In 1929 Knudson (*13*) reported growing seedlings of *Calluna vulgaris* for more than 2 months without the formation of mycorhiza. The plants rooted normally. Seed were surface-sterilized with calcium hypochlorite solution and planted on nutrient agar in test tubes. The agar slants remained sterile except one series in which the seed contained a considerable amount of chaff. All the cultures in this particular series developed an *Alternaria*, which was unable to penetrate into the roots. *Phoma* did not appear in any of Knudson's cultures, and no attempt was made to isolate the endophyte from roots of naturally infected plants.

Rayner immediately criticized Knudson's work, claiming that he used imperfect sterilization methods and then overlooked infection in his seedlings because of faulty technique. She stated (*22, pp. 383–384*):

> In imperfectly sterilised cultures [i. e., those in which the *Phoma* presumably occurring in the seed coats is not killed] in an aseptic rooting medium typical mycorrhiza is not formed; mycelium is casual in distribution, often very sparsely developed, and may be extremely difficult to put in evidence in the earlier stages of growth. * * * The early stages of infection in cultures such as those described show no hyphal complexes within the root cells * * *. The two phenomena—infection of the seedling at germination and the formation of mycorrhiza—are distinct; the former is invariable, the latter conditioned by the nature of the rooting medium * * * Presumably, by "root infection" Knudson understands the formation of the characteristic hyphal complexes found in typical *Calluna* mycorrhiza. In my experience, these are never formed in a sterile agar rooting medium of the kind used * * *. Knudson's conclusions tend to confirm those reported by Christoph because he has employed a similar technique and does not appreciate the true character of seedling infection as distinct from mycorrhiza formation.

Further support for the nitrogen-fixing ability of *Phoma radicis callunae* was reported in 1929 by Rayner and Smith (*24*). In the course of a physiological investigation of the fungus it was found that both the fungus and *Calluna* seedlings thrived better when nitrates and other forms of combined nitrogen were left out of the nutrient media. Strains of the endophyte were said to exhibit a high degree of specialization toward their respective host plants. On the subject of mycorhiza formation it was again stated (*24, p. 287*) that "(* * * roots although infected by mycelium do not develop functional mycorrhiza in a sterilised substrate)."

Investigating the nutrient requirements of the cranberry (*Vaccinium macrocarpon*) in 1931 and 1932, Addoms and Mounce (*1, 2*) concluded that the mycorhizal fungus alone was not able to supply the plant's demand for nitrogen. Rooted cuttings were grown in sand in open pots. Fungus mycelium, presumably of *Phoma radicis*, was observed in above-ground plant parts, including fruit and seed, as well

as in roots. There appeared to be a positive correlation between the amount of mycelium present and vigor of growth in the host plant. Isolation of the fungus was not attempted.

Two important contributions to the subject appeared in 1933. Knudson (*14*) repeated his experiments with *Calluna* with greater precision and presented new evidence that calcium hypochlorite effectively sterilized the seed. Using Rayner's microscopic technique, he was again unable to detect mycelium of any kind in seedlings grown on agar. Root formation was completely inhibited on such agar substrates as potato dextrose and those containing peptone, but seedlings rooted normally on favorable media, i. e., the rooting response was obviously controlled by the composition of the substratum. Again *Phoma* failed to appear in any of the cultures. Friesleben (*9*) isolated from the roots of *Vaccinium myrtillus* a mycorhizal fungus that reproduced the "knot" type of mycorhiza in seedlings grown from surface-sterilized seed. The fungus, which failed to fruit, was tentatively called *Mycelium radicis myrtilli*. The plants were grown in a peat-sand mixture, in flasks that were sterilized and handled aseptically throughout the experiment. Employing Rayner's technique, Friesleben was unable to find the attenuated form of infection in his "sterile" plants. In one important respect his results confirmed those of Rayner, namely, that seedlings in the fungus-free flasks failed to produce roots and soon died.

Using the same technique as before, Friesleben (*10*) in 1934 isolated four root fungi from three *Vaccinium* species (two strains from *V. myrtillus* and one each from *V. vitis-idaea* and *V. uliginosum*), each of which induced formation of true mycorhiza not only in its original host plant but also in the other three species of *Vaccinium* and in *V. oxycoccus* as well. Stimulation to root and shoot growth accompanied synthesis in all cases. None of the fungi fruited. Still more important, Friesleben found that the stimulus to root formation in sterilized peat was brought about equally well by several nonmycorhiza-forming fungi isolated from soil surrounding roots and from surfaces of vegetative parts of native plants. A culture of Rayner's *Phoma radicis callunae* parasitized *V. oxycoccus* but reacted toward *V. vitis-idaea* in the same manner as the other nonmycorhiza-forming fungi. These discoveries compelled Friesleben to abandon the idea of specific and obligate symbiosis in *Vaccinium* and to hold instead that root suppression is due to an unfavorable condition in the rooting medium, which may be corrected by the presence of various fungi whether or not they are mycorhiza forming. He pointed out that fungi might alter the substratum either by secreting substances needed by the higher plant or by breaking down toxic substances produced during sterilization.

A comprehensive series of experiments with ericaceous mycorhiza was reported by Friesleben in 1935 (*11*). Seedlings of 21 species in 13 genera, representing all except 2 tribes of the Ericaceae, were grown in sterilized peat, and except for the tribe Arbuteae all the species exhibited growth repression similar to that found earlier (*10*) in *Vaccinium* species. The repressive effect disappeared when the sterilized peat was inoculated with living cultures either of the root fungi previously isolated from *Vaccinium* spp. (*10*) or of certain other nonmycorhiza-forming soil fungi. Mycorhiza was produced by the *Vaccinium* endophytes in most of the species. Seedlings in asymbiotic

cultures on favorable nutrient agar rooted and grew well, but not quite so vigorously as in inoculated sterilized peat. On the other hand, repressive effects similar to those of sterilized peat developed in cultures on the following media: (1) Ether, alcohol, and water extracts from peat; (2) agars containing peptone, malt extract, and potato; and (3) extracts from fungus mycelium. Friesleben concluded that the stimulating effect of fungi on heath plants is brought about by inactivation, destruction, or absorption of repressive substances in the rooting medium, rather than by secretion by the fungi of substances stimulating to the higher plant.

In the mass of conflicting evidence and opinion outlined above, investigators have agreed that ericaceous plants generally possess mycorhizal fungi in a form variously referred to as "hyphal complex," "knot," or "Hyphenknäuel," because of the typical structures produced by the fungi in root cells. Fungi isolated from these "hyphal complexes" have been found to reproduce similar structures under experimental control in *Calluna vulgaris* (by Christoph), *Vaccinium corymbosum* and *V. pennsylvanicum* (by Doak), and *V. myrtillus*, *V. vitis-idaea*, and *V. uliginosum* (by Friesleben). Friesleben's *Vaccinium* endophytes also produced the hyphal complex type of mycorhiza in 10 additional genera of Ericaceae, including *C. vulgaris*. The endophytic fungi isolated by these investigators failed to produce spores when grown in pure culture. Plants grown in the absence of the endophytes apparently developed normally when certain other necessary conditions were provided.

On the contrary, there has been lack of agreement regarding the role attributed to *Phoma radicis*. Rayner's views have been repeatedly and clearly set forth, as indicated by excerpts in the foregoing review. Two of the fundamental claims she makes for the fungus have been placed in a doubtful category by other workers, since (1) root suppression has been attributed (by Knudson and by Friesleben) to factors inherent in the rooting medium rather than, as Rayner postulates, to lack of *Phoma* in the plants and (2) the actual existence of systemic infection in the form of "fine hyphae" has been openly questioned. Confirmation of the latter phenomenon has come only from observers (Dufrénoy, Rivett, Addoms and Mounce) who have examined material microscopically but have not attempted to grow the fungus and host apart from one another. Those who have isolated endophytes from roots (Christoph, Doak, and Friesleben) or who have grown plants for the specific purpose of determining whether mycorhiza is indispensable (Stahl and Knudson) have not confirmed it. The widely circulated view that *P. radicis* is the fungus causing true mycorhiza in the Ericaceae is not based on experimental proof, since every strain of *Phoma* so far isolated has failed to produce the hyphal complex form of mycorhiza under controlled conditions (Ternetz; Rayner; and Friesleben (*10, p. 449*)).

## SCOPE OF THE PRESENT INVESTIGATION

The present paper reports isolation of root endophytes from four ericaceous species—*Vaccinium macrocarpon* Ait., *V. canadense* Kalm., *Chamaedaphne calyculata* (L.) Moench, and *Ledum groenlandicum* Oeder.—and a method of synthesizing mycorhiza in a sterilized agar medium. Although the endophytes from the four host plants have

not fruited, marked growth differences in culture leave little doubt that the fungi are specifically distinct, and none of them resembles *Phoma radicis* as described in the literature or as compared with a culture of *P. radicis callunae* Rayner, obtained from the Centralbureau voor Schimmel Cultures in Baarn, Netherlands. Originating from three different host genera, all four fungi produce hyphal complex infections in roots of cranberry seedlings (*V. macrocarpon*) grown on agar under pure-culture conditions. The freedom with which the fungi penetrate roots, as well as the rooting response of seedlings, is shown to be markedly influenced by the constitution of the substrate.

## ISOLATION OF THE ENDOPHYTES

The cranberry endophyte was isolated from roots of seedlings grown in the greenhouse in Washington, D. C., in peat brought from a commercial cranberry planting in Wisconsin the previous fall and kept moist until used. Seeds were planted in January and February 1932. Seedlings started emerging about 3 weeks after planting, and a month later some of the roots on most plants were typically infected with the hyphal complex form of mycorhizal fungus.

Isolations were made without previous surface sterilization of the roots. Roots were thoroughly washed in sterilized water with a soft camel's-hair brush and layers of epidermis one cell in thickness were stripped from them with No. 12 steel needles. After the positions of favorably located infected cells had been carefully noted under the compound microscope, the strips were transferred to a watch glass, where the epidermal tissue was further dissected into pieces of some half dozen cells or less, containing a minimum number of infected cells. Five or six of these pieces were transferred to the surface of a thin layer of nutrient agar on a cover glass, covered with a small drop of clear water agar, and then inverted over a Van Tieghem cell with a drop of sterile water in the bottom. In the most favorable preparations the pieces of epidermis lay parallel to and near the cover glass, completely embedded in agar, so that growth from the mycorhizal cells could not only be watched under a 4-mm objective but could also be photographed in situ with a fair degree of success.

Out of a large number of slides prepared in this manner, most of the tissue pieces remained sterile, a few were evidently contaminated, and 16 yielded pure cultures of the endophyte. As observed under the microscope, the first indication of growth in the mycorhizal cells was an increase in the mass of internal hyphae, sometimes to the extent of distending the cell walls considerably before the hyphae broke through. Some 40 hours or so after the cultures were started, hyphae began to pierce the walls and to grow into the agar at an extremely slow rate. The slides were held under observation for at least 6 days, after which the developing fungus growths were transferred to test tubes of nutrient agar. Plate 1, *A* to *C*, illustrates the development of hyphae in one such preparation that contained three "mycorhizal cells" (indicated as *a*, *b*, and *c*). In plate 1, *A*, photographed 44 hours after the slide was prepared, a hypha had just emerged from cell *a*; there was no growth from the other two cells. In *B*, 92 hours old, a second hypha had made its appearance, coming from cell *b*. *C* shows the condition at 139 hours, with cell *c* greatly swollen by the development of internal hyphae. A short time later, numerous hyphae grew out

from cell *c* and the entire piece was transferred to a test tube. *D* shows another preparation photographed when 6 days old, in which several hyphae had emerged from the torn end of the single mycorhizal cell.

The same method of isolation was followed with the other three host species except that, instead of using roots of seedlings, mature plants were dug, all fine rootlets were removed, and the plants were reset in the same soil. Isolations were made from newly formed rootlets as soon as mycorhiza formation was evident. This part of the work was carried out during the summer of 1932 in Wisconsin, where equipment was lacking to make photographic records; however, the development of hyphae was very carefully noted under the high powers of the microscope. *Vaccinium canadense* gave exceptionally satisfactory preparations, as the infected epidermal cells separated easily and two transfers of completely free single cells were obtained, each of which yielded a culture of the fungus. A total of 8 isolations of the *V. canadense* endophyte, 14 of the *Chamaedaphne* endophyte, and 2 of the *Ledum* endophyte were secured. All fungi from each host plant were identical in culture.

## CULTURAL CHARACTERISTICS

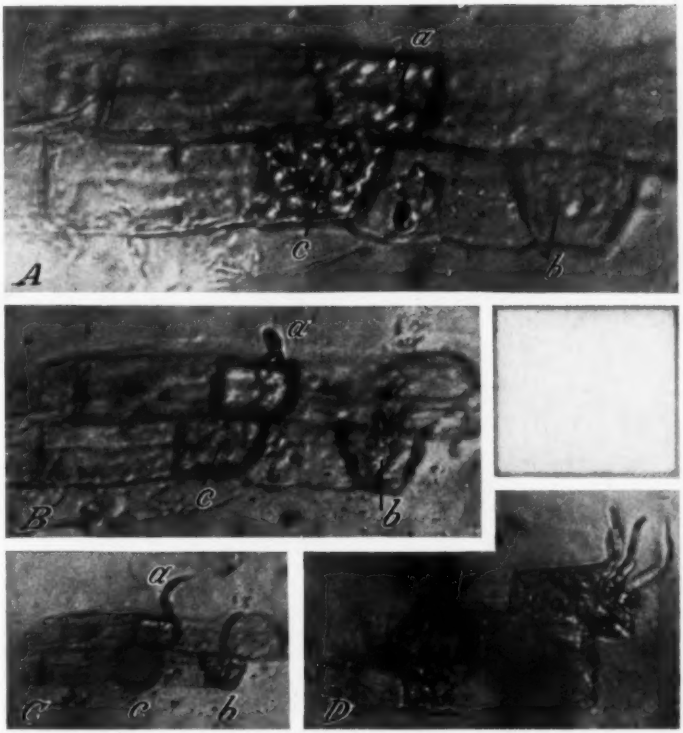
The cultures were subsequently grown on a wide variety of media, including most of the common laboratory sorts, and were subjected to various light and temperature conditions in unsuccessful attempts to induce sporulation. Aside from the fact that all four fungi have septate hyphae, no morphological characters have been developed that would help to determine their systematic relationships. The most prominent characteristic of the fungi as a group is their extremely slow rate of growth, the cranberry fungus having a radial growth of about 0.25 mm per day at room temperature on most media; the blueberry (*Vaccinium canadense*) fungus, about 0.10 mm; the *Ledum* fungus, about 0.20 mm; and the *Chamaedaphne* fungus, about 0.40 mm.

Individual hyphae of the cranberry fungus are invariably hyaline. On some media they remain relatively slender and separate, with the definitely self-limiting colony submerged (pl. 2, *A*, *c*), and they are cartridge buff [3] in color. On other media, notably on onion and beer-wort agars, the colony becomes semistromatic with cells greatly swollen and densely packed with oil globules, the fungus tissue eventually piling up on the surface and breaking into numerous folds and fissures (pl. 2, *B*, *c*). In the latter condition and in older cultures on some other media the colony assumes a chamois or Natal-brown color.

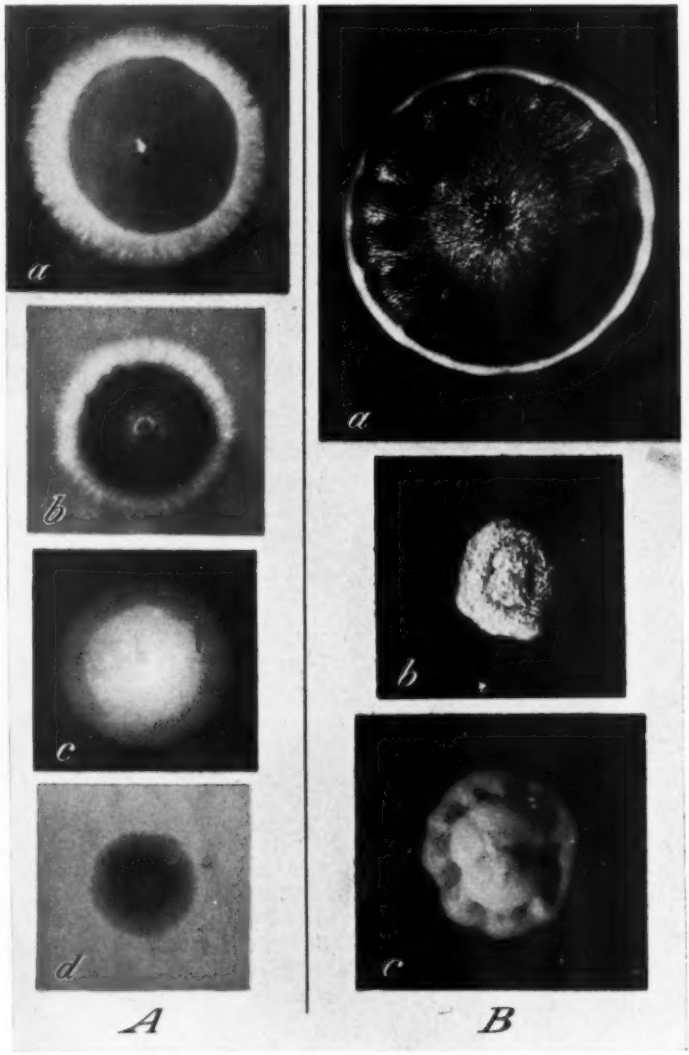
The blueberry (*Vaccinium canadense*) fungus presents an extreme contrast. Here the colony is dark ivy green to olivaceous black, and the individual hyphae are smoky, even showing a dark tinge in hyphal walls when forming "knots" in the lumina of cranberry root cells. The colony does not grow as large as the cranberry fungus (pl. 2, *A*, *d*). The major portion of the colony is submerged, but it is usually covered with a velvety layer of erect aerial hyphae about 0.5 mm in length. Oil globules occur in abundance on most media.

The *Chamaedaphne* endophyte has hyaline hyphae that on some media become dark-colored with age. The colony on most media is dark-colored, and a thin layer of aerial hyphae above darker sub-

[3] Color names used are according to Ridgway (*85*).




Cranberry mycorhizal fungus isolations. *A*, Culture No. 24, 44 hours after being placed in hanging drop of agar. The eight-celled piece of root epidermis contains three hyphal complex cells (*a*, *b*, *c*). A hypha has emerged from cell *a*; no growth from cells *b* and *c*. × 750. *B*, Culture No. 24, 92 hours old. Hypha at *a* has elongated, a second hypha has emerged from cell *b*, and cell *c* is beginning to enlarge from internal growth of hyphae. × 500. *C*, Culture No. 24, 139 hours old. Cell *c* is greatly enlarged by internal growth of hyphae. × 270. *D*, Culture No. 25, 6 days old. Hyphae emerging from torn end of single mycorhizal cell. × 240.




Mycorhizal fungus colonies: *A*, 44 days old on modified McArdle agar; *B*, 47 days old on onion agar; *a*, *Chamaedaphne* endophyte; *b*, *Ledum* endophyte; *c*, cranberry endophyte; *d*, blueberry endophyte. Note particularly the difference in type of growth between *a* and *b* on onion agar and *a* and *b* on McArdle agar. × 1½.

merged mycelium produces a mass effect of deep olive gray to olivaceous black. On some media the color is deep mouse gray, with a margin almost colorless (pl. 2, *A*, *a*, and *B*, *a*). The hyphae penetrate more deeply into the agar than do the hyphae of the two fungi just discussed, and in test tubes the colony eventually covers the entire agar surface; that is, it is not self-limiting. Oil is not ordinarily formed in the hyphae.

The *Ledum* fungus hyphae are hyaline at first and remain so on some media while becoming dark with age on others. The colony is mummy brown to fuscous black practically to its margin. On most media the mycelium is submerged, but occasionally aerial tufts of pale gull-gray to dark olive-gray hyphae are produced (pl. 2, *A*, *b*, and *B*, *b*) The colony rarely extends to the edge of the agar surface. Oil is formed abundantly on some media and is entirely lacking on others. The *Ledum* and *Chamaedaphne* endophytes bear more resemblance to each other than to any of the other fungi, but exhibit well-defined differences on every medium so far used (pl. 2, *A* and *B*).

The cultures have been maintained since 1932 on a modification of the agar used by McArdle (*15*, *p. 299*, *footnote*), made up as follows:

| | *Grams* | | *Grams* |
|---|---|---|---|
| Agar | 15 to 20 | $Ca(NO_3)_2$ | 0. 50 |
| Maltose | 2. 0 | $KH_2PO_4$ | . 25 |
| Dextrose | 2. 0 | | *Cubic centimeters* |
| Soluble starch | 2. 0 | | |
| $MgSO_4$ | . 50 | $H_2O$ (distilled) | 1, 000 |

To this formula a small amount of peptone is sometimes added, to the advantage of the fungi but spoiling the usefulness of the culture as a source of inoculum because of the repressive effect of the peptone on seedling cranberry roots. Various sugars have been satisfactorily substituted for those named, and ammonium sulphate $(NH_4)_2SO_4$, may partly or completely replace the calcium nitrate, $Ca(NO_3)_2$, as a source of nitrogen.

## SYNTHESIS EXPERIMENTS WITH THE CRANBERRY MYCORHIZAL FUNGUS

### METHODS AND OBJECTIVES

After numerous attempts had been made to obtain root infection in cranberry seedlings growing in agar, the following method was found to give consistently good results: Sterile seed was planted in test tubes on 0.85- or 1-percent agar, made with distilled water, to which no nutrients of any kind were added. Sterile seed was obtained either by transferring seed directly from surface-sterilized sound berries or by treating cleaned seed with calcium hypochlorite solution (*10*, *14*, etc.) for 30 minutes or longer. In this connection it may be mentioned that cranberry seed germinate with much greater regularity if permitted to remain in the fruit until January at a temperature sufficiently low to keep the berries in prime condition (*4*, *p. 10*). Both methods of sterilization of seed fail to meet Rayner's view that it is not possible to eliminate endosperm-borne mycelium of *Phoma radicis* from the seeds. This matter is considered at length in a later section, but it may be pointed out here that literally hundreds of plantings have been made on water agar and on nutrient agars

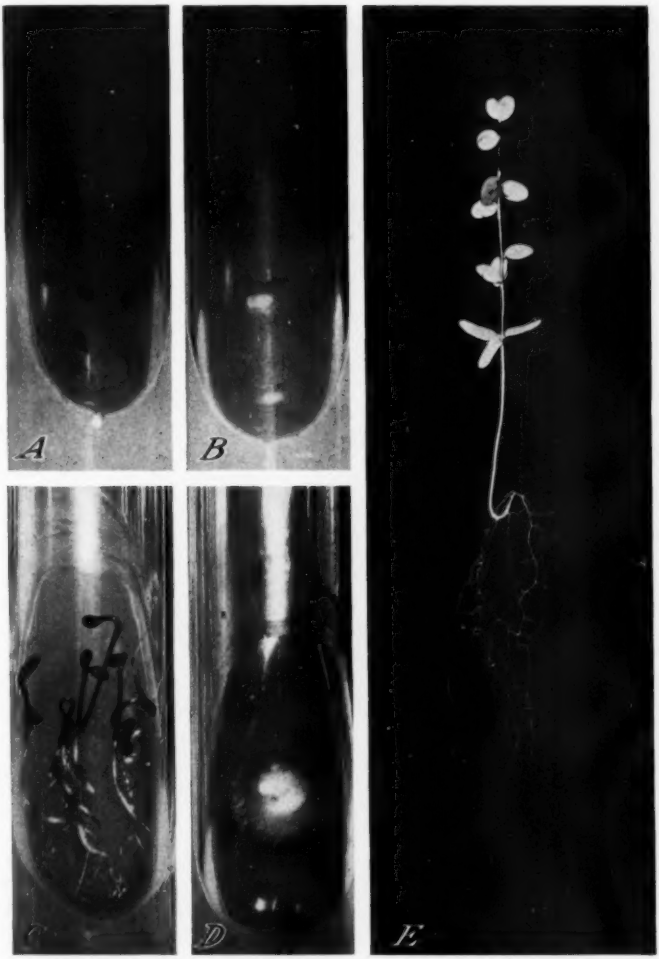
favorable to the growth of the cranberry root endophyte without once encountering *Phoma radicis* in culture (pl. 3, *A* to *D*).

As the seed germinates the roots penetrate the agar freely and in most cases remain slender and bright-colored, branch naturally, and appear to be normal and healthy in all respects. The plants grow for 3 or 4 months without showing marked signs of nutritional deficiences, reach an average height of about 4 cm, and produce six or more true leaves. At the end of the period they differ from plants grown in soil mainly in having somewhat longer internodes, fewer and slightly smaller leaves, and a much reduced root system. If transplanted to soil from water agar while actively growing, the seedlings will thrive as well as soil-planted seedlings of the same age. In the tubes the plants may remain alive and green for a year provided the agar does not dry out. Exposed roots redden in bright sunlight. Plate 3, *E*, illustrates an average 16-weeks-old plant grown in water agar.

An occasional lot of distilled-water agar proves to be somewhat unsatisfactory. Here the roots cease growing, after penetrating a short distance into the agar, and send out numerous abortive branches, and the entire root system loses its bright, clear color, turning brown or black. The aerial portion of the plant is not affected, although there is a tendency to produce adventitious roots above the agar. This is apparently a mild form of toxicity due to the medium. More severe cases of toxicity brought about by adding various substances to the agar are discussed later.

The seedling cultures may be inoculated with the mycorhizal fungus at the time the seed is planted, or if it is preferred to test the sterility of the seed the operation may be delayed until after the seed has germinated. Inoculations are made simply by spreading liberal quantities of crumbled fungus tissue from stock cultures (p. 819) among the seeds or plants (pl. 4, *A*). Hyphae from each piece of inoculum grow remarkably long distances into the nutrient-free medium. Mycorhiza formation takes place almost immediately after the slowly advancing hyphae come in contact with the roots. The hyphae follow the roots into the agar far beyond the depth to which they would otherwise penetrate, and continue to form mycorhiza as they advance.

The type of infection that develops in the water-agar cultures appears to be identical with that in natural cranberry mycorhiza. After entering an epidermal cell (pl. 4, *B*), the hypha coils and branches freely within the lumen (pl. 4, *B* and *C*) until the enveloping mycelial mass finally fills the cell completely (pl. 5, *B*). The end result is digestion or disintegration of the mycelial mass within the cell exactly as it occurs in nature (*20, pp. 268–269*). The prevailing pattern of infection consists of infected epidermal cells, either single or in small groups, separated by larger areas of fungus-free tissue (pl. 5, *A* and *B*). Internal hyphae have been observed to pass from cell to cell (pl. 5, *C*), but apparently the plant inhibits an extensive spread of the fungus in this manner; the completely infected epidermal areas often encountered probably result from separate external infections. The fungus has never been observed to penetrate more deeply into the root than the epidermal layer. Infection is strictly limited to root tissue, never occurring above the root-stem transition zone, despite the fact that external mycelium frequently advances well up the stem under agar-culture conditions.




*A*, Sterile cranberry seed planted on modified McArdle agar February 5, 1934. *B*, Tube of same agar inoculated with cranberry root endophyte. *C* and *D*, Same cultures as in *A* and *B*, 29 days later. Note that seed culture remained sterile although medium is favorable to growth of endophyte. *E*, Average 16-weeks-old cranberry seedling grown on sterile distilled-water agar. × 1½.

*A*, Culture of cranberry seedlings on distilled-water agar, planted January 15, 1934, inoculated with the cranberry root endophyte January 29; photographed February 28. Plant *a* was removed March 10, when photomicrographs of mycorhiza, reproduced in plate 5, *A* and *B*, were made from its roots. × 1½. *B*, Cranberry mycorhizal fungus entering epidermal cells of cranberry seedling root in distilled-water agar culture; seed planted January 15, 1934, inoculated January 29; photographed March 13. × 963. *C*, Epidermal cell almost filled by mycorhizal mycelial complex. From same culture as *B*, photographed March 24, 1934. × 963.

Several questions immediately arise in regard to the significance of infections obtained in the manner described. Do they provide conclusive proof that the fungus is in fact identical with the endophyte that produces similar structures under natural conditions? In other words, is this true mycorhiza formation? Or is it more probable that the plants are partly starved when grown on nutrient-free agar and consequently have lost their ability to resist invasion by the fungus? Is the plant either injured or benefited as a result of the infection? Will fungi from other sources produce similar formations in the roots? Is *Phoma radicis* present systemically in both inoculated and uninoculated plants? If *Phoma*, rather than the fungus isolated by the writer, is the true endophyte, why does it not produce the "mycelial complexes" under these conditions as the cranberry fungus does? In an attempt to answer these and related questions numerous experiments have been conducted.

Agar rather than sterilized soil was used for the inoculation experiments because it appeared to offer certain advantages over soil, particularly in respect to the ease of varying the nutrient composition of the substrate and of observing the behavior of fungi, whether introduced intentionally or accidentally. The stock culture medium (p. 819) that had proved favorable to the fungus was first tried, with disappointing results. Roots coming in contact with the agar did not grow normally, but soon turned brown and failed to penetrate into the medium. (Compare pls. 3, *C*, and 4, *A*.) In the inoculated series the fungus enveloped the surface-lying roots with a dense weft of hyphae but only on rare occasions penetrated into epidermal cells to produce characteristic hyphal complexes. These few instances of infection, however, closely resembled mycorhiza and made it appear that the true endophyte was being used, but under conditions not conducive to abundant infection. Proceeding next on the assumption that the fungus might obtain food materials from roots if not available in the culture medium, nutrient-free agar was tried, resulting in immediate and at times extremely heavy infection as noted above.

### RESULTS OF EXPERIMENTS

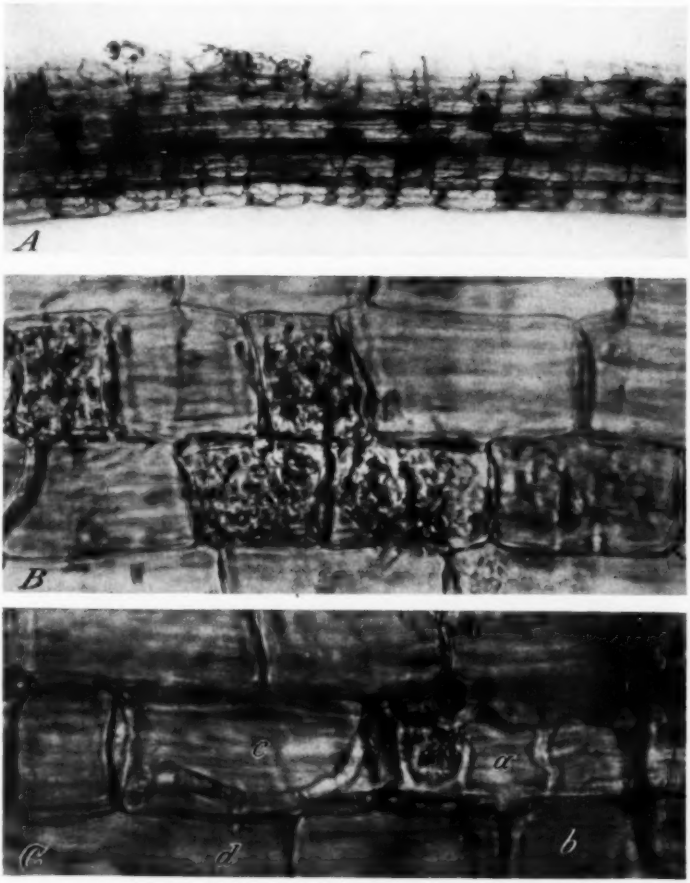
In a series of water-agar cultures started in the spring of 1935, 6 seeds were planted in each of 284 test tubes, 142 of which were inoculated with the cranberry endophyte. Frequent microscopic examination showed that plants in the inoculated series were invariably mycorhizal while those in the uninoculated checks lacked mycorhiza. At the end of 16 weeks 750 plants remained in the inoculated series and 767 in the checks. At this time the average height of 25 random plants in the inoculated series was 41 mm and in the uninoculated series it was 39 mm; both lots had an average of 7 true leaves. All the plants were then removed, with root systems intact, and were thoroughly air-dried and weighed. The weight per plant in the inoculated series was 3.64 mg and in the uninoculated series 3.63 mg. The seed reserve obviously must have supplied practically all mineral nutrients used by the plants grown under both conditions; the fungus did not seriously deplete the store; and nitrogen availability and starch formation must have been about equal in the two lots. It appears, then, that the fungus was neither injuriously parasitic nor beneficial to the plants under the conditions of the experiment.

Some curious results were obtained in another series of plantings in 1935, in which an attempt was made to find out whether the fungus derived certain substances or elements from the roots. With modified McArdle agar as a base, one after another of the chemical compounds was omitted, and by making substitutions in the formula, a single element at a time was eliminated. The formulas were made up as shown in table 1, with 15 g of agar and 1,000 cc of distilled water added to each except to the nutrient-free agar.

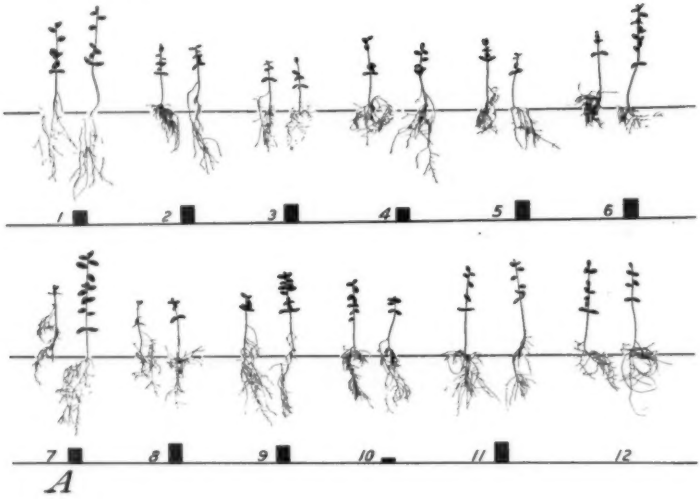
The cranberry fungus was grown for 2 months in a tube from each lot, after which transfers were made from the edges of the colonies to fresh lots of the same agars. The diameters of the colonies on the subcultures were measured when the colonies were 19 days old; the results are shown in plate 6, *A*. There was definite growth in every lot, though the sparse development of hyphae in No. 1 and the reduced rate of growth in No. 10 were decidedly subnormal. Growth was slightly subnormal in all others except Nos. 2 and 9.

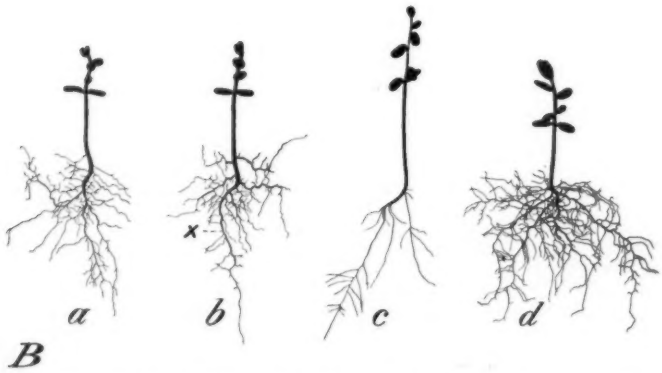
On February 5, seed from surface-sterilized berries were planted, five or six to the test tube, on the agars described above. Germination was irregular. Half of the tubes were inoculated with standard agar cultures of the cranberry fungus after the seed germinated. The other half were held as checks. The tubes were capped with waxed paper and kept in the greenhouse for 16 weeks. Roots from both inoculated and check lots were examined at frequent intervals for fungus infection. At the conclusion of the period 25 plants from each lot were measured, and all plants were carefully removed, air-dried for 4 months, and weighed.

Marked differences in growth were evident from the beginning, showing in such respects as height, color, and thickness of stems; size, number, and color of leaves; numbers and branching of roots; penetration of roots into the agar; and formation of adventitious roots that grew along the glass surface opposite the agar. Notes are summarized in table 2 and charted in plate 6, *A*.




*A*, Root from plant *a* (pl. 4, *A*), photographed March 10, 1934, showing distribution of infected (dark-colored) epidermal cells. × 167. *B*, Mycorhiza from plant *a* (pl. 4, *A*), photographed March 10, 1934; in four infected cells the mycelium is in early stages of digestion; in lower right the hyphal coils have not quite filled the cell. × 750. *C*, Early stages of infection in water-agar culture; hyphae branching and beginning to coil in cell *a* and passing through cell walls into *b* and *c*; a branch from *c* has started into *d*; photographed March 23, 1934. × 750.




*A*, Average plants in the 1935 agar-culture series (tables 1 and 2); in each pair, plant on left is from inoculated culture, on right from uninoculated culture; blocks represent relative diameters of cranberry mycorhizal fungus colonies on the respective agars (1–12) when 19 days old; individual plants were photographed and the prints were inked over after being assembled, thus making roots appear considerably heavier than they actually were. × approximately ⅛. *B*, Cranberry seedlings 60 days from date of planting, grown in (*a*) cork-sand mixture, not inoculated, not mycorhizal; (*b*) cork-sand mixture, inoculated with cranberry mycorhizal fungus, all roots above *x* infected; (*c*) distilled-water agar in test tube, inoculated with mycorhizal fungus, mycorhizal; (*d*) Wisconsin peat, mycorhizal; traced root by root under enlarging camera; diameter of roots enlarged for illustration. × Approximately ¾.

TABLE 1.—*Constituents*[1] *of media used for the agar-culture series of cranberry seedlings in 1935*

| Lot No.[2] | Nutrient omitted | $MgSO_4$ | $Ca(NO_3)_2$ | $KH_2PO_4$ | Carbohydrates | | | $MgCO_3$ | $(NH_4)_2SO_4$ | $CaCl_2$ | $NH_4H_2PO_4$ | $K_2CO_3$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Maltose | Dextrose | Soluble starch | | | | | |
| | | *Gram* | *Gram* | *Gram* | *Grams* | *Grams* | *Grams* | *Gram* | *Gram* | *Gram* | *Gram* | *Gram* |
| 1 | Carbohydrates | 0.5 | 0.5 | 0.25 | | | | | | | | |
| 2 | $MgSO_4$ | | .5 | .25 | 2.0 | 2.0 | | | | | | |
| 3 | $Ca(NO_3)_2$ | .5 | | .25 | 2.0 | 2.0 | 2.0 | | | | | |
| 4 | $KH_2PO_4$ | .5 | .5 | | 2.0 | 2.0 | 2.0 | | | | | |
| 5 | Sulphur | | .5 | .25 | 2.0 | 2.0 | 2.0 | 0.5 | | | | |
| 6 | Magnesium | | .25 | .25 | 2.0 | 2.0 | 2.0 | | 0.25 | | | |
| 7 | Calcium | .5 | | .25 | 2.0 | 2.0 | 2.0 | | .5 | | | |
| 8 | Nitrogen | .5 | | .25 | 2.0 | 2.0 | 2.0 | | | 0.25 | | |
| 9 | Potassium | .5 | .5 | | 2.0 | 2.0 | 2.0 | | | | 0.25 | |
| 10 | Phosphorus | .5 | .5 | | 2.0 | 2.0 | 2.0 | | | | | 0.25 |
| 11 | | .5 | .5 | .25 | 2.0 | 2.0 | 2.0 | | | | | |

[1] Made up with 15 g of agar and 1,000 cc of distilled water.
[2] The check (lot No. 12, table 2), from which all nutrients were omitted, consisted of 1-percent distilled-water agar as described on p. 819.

TABLE 2.—*Data on agar-culture series of cranberry seedlings in 1935* [1]

| Lot no. and description | Treatment | Plants | Height of stems | True leaves | Weight of plants | Characters of— | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Stems | Leaves | Roots |
| | | *No.* | *Mm* | *No.* | *Mg* | | | |
| 1 (lacking carbohydrates). | Inoculated | 191 | 42 | 9 | 4.1 | Green | Large, green, vigorous | Mostly clear, long, slender, not stubby, penetrating agar readily, moderately branched. Mycorhiza formed freely in some plants, scarce or lacking in others. |
| | Not inoculated | 210 | 43 | 8 | 3.8 | do | do | Clear, long, slender, moderately branched, penetrating agar freely. No mycorhiza. |
| 2 (lacking $MgSO_4$) | Inoculated [2] | 215 | 29 | 7 | 4.8 | Thick, reddish brown | Small, reddish | Light reddish brown, much branched, mostly penetrating agar about 10 mm, few individuals more deeply. Some mycorhizal cells in roots in agar. |
| | Not inoculated | 214 | 29 | 6 | 3.9 | Reddish brown | Medium size, dark green | Colored but not as brown as in inoculated plants; not stubby, rarely penetrating agar. No mycorhiza. |
| 3 (lacking $Ca(NO_3)_2$) | Inoculated | 187 | 27 | 4 | 4.2 | Thick, reddish brown | Small, green | Light brown, moderately branched, mostly aerial or on glass, some penetration of agar, tendency toward stubbiness. No true mycorhiza, but fungus sometimes penetrated cells and occasionally formed a few coils. Both internal and external hyphae of huge diameter and gorged with oil. |
| | Not inoculated | 182 | 25 | 5 | 3.7 | Thick, dark red above cotyledons. | do | Red, short, moderately branched, penetrating agar slightly, not stubby. No mycorhiza. |
| 4 (lacking $KH_2PO_4$) | Inoculated | 213 | 38 | 7 | 4.7 | Thick, red above cotyledon. | Medium size, green | Light reddish brown, very little penetration of agar, most growth and branching aerial or on glass, fairly long. Occasional mycorhizal cells formed. |
| | Not inoculated | 204 | 30 | 7 | 3.8 | Thick | Medium large, green | Light reddish brown, tendency to stubbiness, slight penetration of agar, roots mostly aerial or on glass, medium long. No mycorhiza. |
| 5 (lacking S) | Inoculated | 147 | 32 | 7 | 4.3 | Red above and below cotyledons. | Small, newer ones with reddish cast. | Light reddish brown, profusely branched, stubby especially in contact with agar, short, very little penetration of agar. Occasionally a mycorhizal cell. |
| | Not inoculated | 186 | 27 | 5 | 3.2 | | Pale, but general appearance of plants good. | Light reddish brown, comparatively long and slender, penetrating agar slightly, no stubbiness. No mycorhiza. |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 6 (lacking Mg) | Inoculated [3] | 175 | 30 | 6 | 3.3 | Thick | Very small, newer ones pale green. | Reddish brown, stubby to tuberculate in contact with agar, short, branching profusely, not penetrating agar. No mycorhiza, but fungus sometimes grew through collapsed epidermal cells. |
| | Not inoculated | 180 | 48 | 10 | 4.5 | Green, slender | Large, rich green | Almost clear, sparse, short, fine and cobwebby, moderately branched, not penetrating agar. No mycorhiza. |
| 7 (lacking Ca) | Inoculated [3] | 72 | 27 | 5 | 3.0 | Red | Dwarfed, newer ones colorless to reddish green. | Reddish brown, very stubby, almost tuberculate in contact with agar, not penetrating, most branches extremely short. No mycorhiza. |
| | Not inoculated [4] | 175 | 48 | 13 | 4.5 | Green to reddish green | Large, dark green | Mostly clear, medium long, moderately branched, penetrating agar to some extent. No mycorhiza. |
| 8 (lacking N) | Inoculated | 201 | 28 | 4 | 4.4 | Thick, straw-colored | Small, light green | Reddish brown, short, occasional slight penetration, stubby in contact with agar. Considerably branched. No mycorhiza but hyphae occasionally grew through collapsed epidermal cells. External hyphae large, gorged with oil. |
| | Not inoculated | 198 | 28 | 5 | 3.7 | Not so thick as in inoculated lot. | Small, deep green | Reddish brown, short, not stubby, moderately branched, penetrating agar only slightly. No mycorhiza. |
| 9 (lacking K) | Inoculated | 187 | 33 | 6 | 3.5 | | Small, newer ones pale | Light brown, considerable variation in growth and branching, not penetrating agar, sometimes tuberculate in contact with agar. No mycorhiza. |
| | Not inoculated | 161 | 39 | 9 | 4.5 | Mostly green, slender | Large, deep green, set close together. General appearance good. | Very light brown to almost clear, moderately long, moderately branched, not penetrating agar. No mycorhiza. |
| 10 (lacking P) | Inoculated | 188 | 37 | 7 | 4.4 | | Large, green | Light brown, medium long, somewhat stubby in and on agar, longer on glass, moderately to profusely branched. No mycorhiza. |
| | Not inoculated | 210 | 27 | 5 | 2.9 | | Very small, yellowish red | Light brown, short, not penetrating agar, longer and moderately branched on glass, not stubby. No mycorhiza. |

[1] Data in columns 4 to 6 are averages based on number of plants (column 3).
[2] Several plants failed to emerge from seed coats.
[3] Seed germinated poorly.
[4] Size and color of plants approached those of soil-grown plants of same age.

TABLE 2.—*Data on agar-culture series of cranberry seedlings in 1935*—Continued

| Lot no. and description | Treatment | Plants | Height of stems | True leaves | Weight of plants | Characters of— | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Stems | Leaves | Roots |
| | | *No.* | *Mm* | *No.* | *Mg* | | | |
| 11 (complete agar) | Inoculated | 170 | 43 | 8 | 4.8 | Red above cotyledons | Medium size, green | Light reddish brown, short and stubby in contact with agar, long and profusely branched on glass, some penetration of agar except in areas occupied by fungus colony. Occasional mycorhizal cells, hyphae sometimes growing through epidermal cells. |
| | Not inoculated | 140 | 41 | 8 | 4.0 | Not so red as in inoculated lot. | do | Light brown, stubby in contact with agar, penetrating slightly; long, slender, and moderately branched along glass. No mycorhiza. |
| 12 (distilled water agar) | Inoculated | 750 | 41 | 7 | 3.6 | Reddish, slender | do | Rather short, not profusely branched, slender, many reddish, others mostly clear, penetrating agar freely and deeply. Mycorhiza formation invariable, infection at times extremely heavy. |
| | Not inoculated | 767 | 39 | 7 | 3.6 | Brownish green, slender | do | Long, slender, sparingly branched, light brown to clear or occasionally red, mostly in agar. No mycorhiza. |

Any attempt to interpret these results evidently must be made by comparing the behavior of plants in nutrient-free agar (No. 12) with that of plants in either complete agar (No. 11) or in the agar in which the plants most nearly approximated normal soil growth (No. 7, uninoculated). The growth differences between inoculated and uninoculated plants on nutrient-free agar, as pointed out, were not measurable. On complete agar the only apparent difference was an increase of 20 percent in weight of the inoculated plants over the checks, while these inoculated plants were but 33⅓ percent heavier than either inoculated or uninoculated plants grown in nutrient-free agar, and were only slightly taller and produced but one more leaf. The plants that grew the tallest stems and the greatest number and largest sized leaves (No. 7, uninoculated) weighed slightly less than those on the inoculated complete-nutrient agar. In the light of these relations it must be concluded that wherever growth was subnormal as compared with plants in the nutrient-free agar, subnormal growth was due to direct injury exerted by the unbalanced substrate and not to deficiencies in the agar. Roots of subnormal plants responded by failure to penetrate the agar freely (in extreme cases becoming tuberculate in contact with it), or by turning brown or red, or by developing adventitiously or branching freely out of contact with the agar. This condition was apparently comparable to the root toxicity found by Friesleben (*9*, *10*, *11*) in using fungus-free sterilized peat, and lends support to Knudson's suggestion (*14*) that the failure of Rayner's agar-culture *Calluna* plants to form roots was probably directly attributable to conditions existing in the substrate. The cranberry plant, by way of contrast, with its remarkable rooting capacity, was able to survive on these unfavorable media for long periods, although stems, leaves, and roots in contact with the agar often showed injury.

Considering the combined effect of media and fungus on the plants, it was found that in three instances (Nos. 4, 5, and 10) the inoculated plants grew better than their uninoculated checks, and in three cases (Nos. 6, 7, and 9) the reverse was true. Where the inoculated plants were superior, there was a trace of mycorhizal infection in two lots, none in the other (No. 10); height of plants and general appearance were inferior to the water-agar cultures, though dry weight per plant was greater, and roots were abnormal. In those lots in which the checks were superior the plants grew decidedly better than the water-agar cultures in two cases (Nos. 6 and 7) and at least equally well in the other (No. 9), and the roots were almost normal in appearance. The plants in No. 1 also produced normal roots both in inoculated and check cultures; in growth they were intermediate between the water-agar and complete-agar cultures; and mycorhiza formation in inoculated plants in this carbohydrate-lacking medium was more abundant than in any other medium except water agar. Taking all these relations into consideration, the following conclusions appear to be justified:

(1) The culture media as made up were injurious to the plants except in Nos. 1, 6, 7, 11, 12, and possibly 9.

(2) The injury was of a toxic nature and was not due to specific nutrient deficiencies.

(3) Presence of the fungus in the cultures tended either to reduce the toxicity (Nos. 4, 5, and 10) or to intensify it (Nos. 6, 7, and 9),

according to the changes in the medium brought about by metabolic activity of the fungus.

(4) Little information was gained regarding the role played by the fungus in the mycorhizal association. However, it was noted that in nutrient-free agar (No. 12) inoculated plants were invariably mycorhizal but no heavier than their nonmycorhizal, uninoculated checks; while in the partial or complete nutrient media Nos. 2, 3, 4, 5, 8, 10, and 11, plants from inoculated cultures gained in weight over corresponding check plants without developing mycorhiza to any extent.

(5) In order to enter the roots freely the fungus must be partially starved, or the roots must be growing satisfactorily in the medium, or both.

(6) Absence of carbohydrates in the medium favors infection.

(7) Cranberry roots are so sensitive to the medium in which they are growing that caution must be exercised in attributing root or plant response to a single factor of the complex plant-medium-fungus association.

Similar changes in root and plant responses are brought about by the cranberry fungus in culture media prepared from extracts of plant substances. For example, corn-meal agar was detrimental to the growth of cranberry seedlings. The plants remained small, leaves were yellowish green, and the roots stopped growing abruptly where they touched the agar, but produced dense mats of reddish-brown branches along the sides of the tubes away from the agar. In the inoculated series plants were nearly twice as tall, leaves were larger and greener, and root development took place almost entirely within the agar. In malt-extract agar roots were incapable of penetrating the uninoculated medium and invariably produced jet-black tubercular or nodular structures where they touched it. Inoculated plants lacked both the blackening and tubercularization, though roots did not penetrate into the agar.

In another series of experiments the ground-cork and sand mixture described by McArdle (*15, footnote p. 301*) was used as a medium instead of agar. Test tubes were filled to a depth of 6 cm, a small amount of the nutrient solution recommended by McArdle was added, and the tubes were autoclaved and inoculated with the cranberry fungus on December 1, 1932. By February 4, 1933, mycelium had penetrated about 1 cm in the tubes. Single sterile plants, which had been planted on standard agar (p. 819) 4 weeks previously as a test for sterility, were then transferred to a number of the tubes. Typical hyphal complex infection was present in roots when the first examination was made on March 14, and before the end of the month most of the roots were heavily infected. Root elongation soon ceased, and the tips enlarged and turned brown. Most of the tubes had dried out when the experiment was concluded April 12. In those tubes that were still moist, the roots had penetrated only 1 cm and lateral roots were short and stubby; but numerous new branches were breaking through just behind the arrested tips, some of which, not more than 3 mm long, were already mycorhizal. Root growth of plants in the uninoculated test tubes was entirely similar, except that in no instance was hyphal complex infection present. It was evident that in both lots some condition of aeration or chemical composition was not entirely favorable to root growth.

Root growth comparable in type and extent to that occurring in normal cranberry soil (peat) was finally attained, but not under pure-culture conditions. On March 3, 1933, ten 2-inch pots were partly filled with the cork-sand mixture and sterilized. Then, without attempting to maintain sterility further, inoculated cork-sand mixture from the December 1 cultures was added, and 10 seeds, transferred directly from surface-sterilized berries, were planted in each pot. Ten similar uninoculated pots were prepared. The two lots were placed some distance apart on a bed of coarse cinders on a greenhouse bench, sprinkled with tap water, and cared for in the routine manner, except that after germination a liberal application of nutrient solution (p. 828) was given every tenth day. Seed germinated and grew similarly in the two lots. On May 2, 12 inoculated and 6 check plants were removed and carefully washed, after which the root systems were accurately traced under an enlarging camera. All roots of the 18 plants were then examined under the microscope for mycorhizal infection.

Mycorhiza formed in all 12 inoculated plants. Practically all roots were infected throughout except near the growing points and occasionally deep in the pots, the general distribution and abundance of mycorhizal cells being very similar to naturally infected roots growing in peat. The roots of the check plants were entirely free from hyphal complexes, though external hyphae of contaminating fungi frequently grew along and around them. A careful comparison of the root systems failed to disclose any consistent differences in amount or type of branching or extent of development between inoculated and check plants. The roots of both lots were bright and clear throughout, exhibiting no symptoms of repression such as had developed when plants were grown in test tubes. A typical inoculated plant is illustrated in plate 6, *B*, *b*; an uninoculated plant grown in cork-sand mixture in *B*, *a*; and, for comparison, an inoculated plant grown on distilled water agar in *B*, *c*, and a normal peat-grown plant of the same age in *B*, *d*.

The final answer to the question whether mycorhiza formation is indispensable to the host will not be known until plants have been carried to the fruiting stage in an aseptic environment in the presence and in the absence of endophytes. This has not been accomplished with the cranberry or with any other species of the Ericaceae. It is submitted that the evidence presented definitely establishes the fungus used in the experiments as the one which produced hyphal complexes in roots from which it was isolated. Given the true endophyte, the next step toward solution of the problem will be to find a sterilizable medium in which both roots and fungus will grow normally under aseptic conditions. The extreme sensitivity of cranberry roots to their environment was conspicuous throughout the experiments just described. Friesleben (*9*, *10*, *11*) has shown that roots of numerous other heath plants will not develop normally in heat-sterilized peat soils.

## SYNTHESIS EXPERIMENTS WITH THE ENDOPHYTES OF CHAMAEDAPHNE, LEDUM, AND VACCINIUM CANADENSE

In several of the agar-culture experiments reported above, parallel series of cranberry seedlings were inoculated with the endophytes of

*Chamaedaphne*, *Ledum*, and *Vaccinium canadense*. It was found that the three fungi were capable of entering epidermal root cells almost as readily as the cranberry fungus and that they produced similar hyphal complex structures, distributed along the roots in a similar manner, without appearing to be injuriously parasitic to the plants. Except in water agar, striking changes in plant and root growth were likewise brought about by inoculating the medium, although the four fungi did not always induce similar responses in the same medium.

Minor differences, correlated in large part with mycelial characteristics, were discernible in the hyphal complexes. For example, the *Vaccinium canadense* complex was always somewhat dark in gross appearance, owing to the color in hyphal walls (pl. 7), and the *Chamaedaphne* (pl. 8) and *Ledum* (pl. 9) complexes were more hyaline than those of the cranberry and *V. canadense* fungi because of thinner hyphal walls. Nevertheless, observed separately, any one of the three would readily pass for typical mycorhizal infection (pl. 10). There appears to be no reason to doubt that in nature these three fungi, and probably others as well, if present in the soil, could enter into mycorhizal union with the cranberry.

Friesleben (*9*, *10*, *11*) reported results similar to these in his experiments, but Rayner and Smith (*24*, *p. 262*) found a high degree of specificity with *Phoma radicis* strains.

## CONCERNING PHOMA RADICIS

Rayner's hypothesis of mycorhizal relations in the heath family has held so conspicuous a place in the literature that her views should be given full consideration in discussing the subject. Briefly summed up, her hypothesis, with its implications, maintains that:

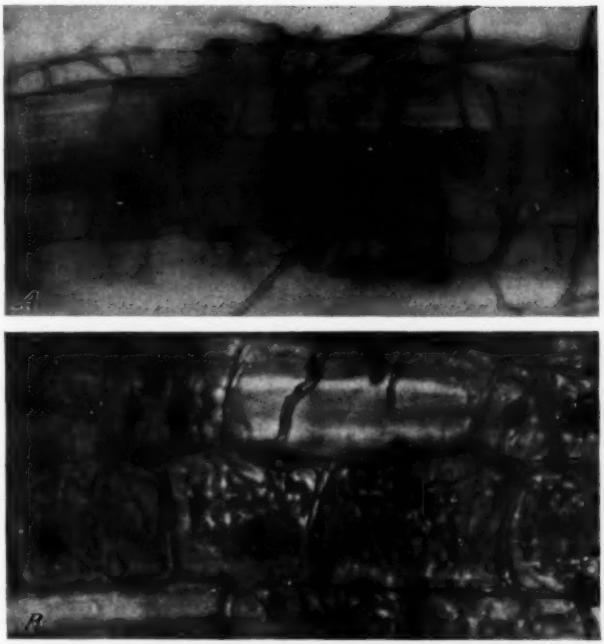
(1) The mycorhizal fungus of the heath family is *Phoma radicis*, a fungus which has specific strains closely restricted to the various members of the family.

(2) This *Phoma* ramifies through the entire plant organism—roots, stems, leaves, flowers, fruits, and seeds. In *Calluna* infection of the seed is limited to the coat, while in *Vaccinium* the mycelium advances into the endosperm.

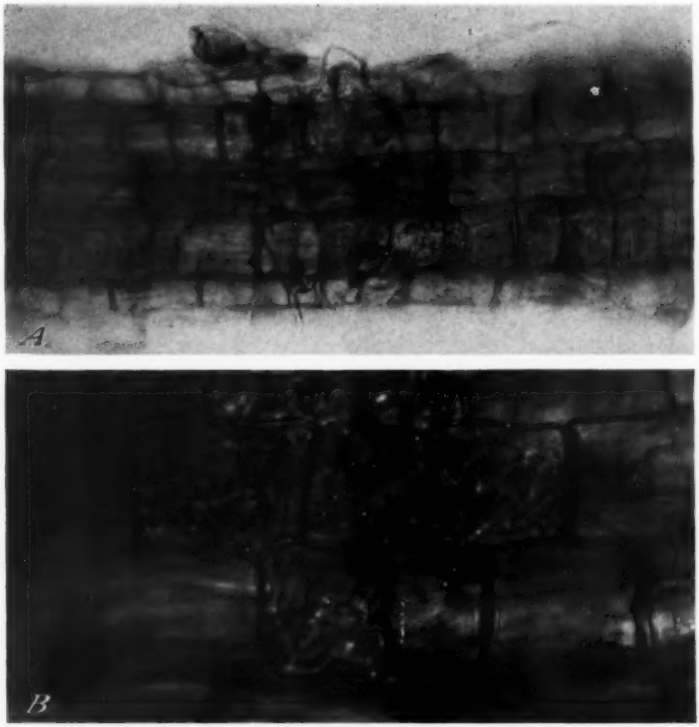
(3) Heath plants therefore cannot escape being infected in nature.

(4) In endosperm-infected species it is impossible to secure plants free from infection by any of the ordinary methods of seed sterilization (*21*, *p. 63*; *22*, *p. 381*).

(5) The mycelium exists in two dissimilar forms in the plant: (*a*) Normal-sized hyphae, easily visible under the microscope, develop intracellularly in roots (hyphal complexes) under suitable environmental conditions; they may arise from the other form of mycelium present in the root, but more often they represent new infections from the soil. (*b*) The hyphae in the systemic form of infection, in roots as well as in above-ground organs, are generally so attenuated in size that a specialized technique is required to make them visible. They form "a continuous network of mycelium in the middle lamellae of walls" (*20*, *p. 282*) and are "closely associated with the cell walls, pass[es] freely in and out of the cells" (*21*, *p. 62*). They sometimes occur in relatively large strands in intercellular spaces of leaves and stems (*17*, *p. 116 et seq.*).



Synthetic mycorhiza in water-agar culture of cranberry, produced by *Vaccinium canadense* endophyte: *A*, Distribution of infected cells, × 375; *B*, detailed structure, × 750.




Synthetic mycorhiza in water-agar culture of cranberry, produced by *Chamaedaphne* endophyte: *A*, Distribution of infected cells, × 375; *B*, detailed structure, × 750.

(6) Seedlings will not produce roots unless infected with *Phoma;* root formation per se is proof of infection. In this sense the mycorhizal relation is obligate to the host.

Each of these points will now be examined critically.

(1) Is *Phoma radicis* the mycorhizal fungus of all heath plants? In the author's experiments, four fungi, microscopically observed to originate from intracellular hyphal complex hyphae in roots of four heath species, reproduced the same type of infection when inoculated into roots of plants grown under completely controlled conditions. The four fungi entered cranberry roots with almost equal facility, and the plants were not injured by their presence so far as could be determined, i. e., there was no evidence of ordinary parasitism. Cultural characters proved conclusively that the four fungi were distinct from one another and from *Phoma radicis.* Christoph (*6*), Doak (*7*), and Friesleben (*9, 10, 11*) reported similar results with other ericaceous species. If the term "mycorhiza" is to be applied to the hyphal complex union of fungus and roots in the heath family, *Phoma radicis* is not the fungus involved in the cases cited, nor is the ability of one fungus species to form mycorhiza restricted to a single host species.

(2) The question of systemic infection by mycorhizal fungi in the heaths has been a matter of controversy, as is indicated in the review of literature. In the present investigations systemic infection in either the check or inoculated plants could not be verified despite thorough search in both fresh and fixed material handled as nearly as possible according to Rayner's published methods. Examination of a large assortment of prepared slides from healthy stems, buds, flowers, and young berries made in connection with a histological study of the cranberry false blossom disease also failed to disclose a single authentic instance of infection by "fine hyphae." Christoph (*6*), Knudson (*14*), and Friesleben (*10*) were likewise unable to find evidence of this type of infection in their material and held that the objects which Rayner called mycelium were most probably artifacts resulting from fixation or staining; but Rayner (*18, 22*) maintained that the inadequate technique of Christoph and Knudson prevented detection of the mycelium. She has not as yet commented on Friesleben's findings.

Some of the writer's material was submitted for examination to Dr. Ruth M. Addoms, who has conducted investigations on cranberry mycorhiza (*1, 2*), and her report is here quoted in part:

> Following are my notes on your cranberry cultures, which I have examined with some care:
>
> (A1) [Water-agar cultures inoculated with the cranberry fungus].—Roots long and red; fungus present externally and in epidermal cells.
>
> (A2) [Water-agar check for above, not inoculated].—No infection; not red.
>
> (B1) [Water-agar cultures inoculated with cranberry fungus, younger culture than above].—Roots red, much branched, infected.
>
> (B2, C2, and D2) [Uninoculated cultures on water agar, serving as checks for B1, C1, and D1].—Roots poorly developed, stems long; no infection.
>
> (C1) [Water-agar cultures inoculated with *Vaccinium canadense* fungus].—Roots not very long and blackish, heavily infected; fungus much coarser and browner than cranberry fungus, with longer cells; some fungus on surface of stem.
>
> (D1) [Water-agar cultures inoculated with *Ledum* fungus].—Roots not very long; definitely infected.
>
> As these notes show, I have not found infection in your check plants. Also I nave not found the fungus within the cells of the stem, although this part of my examination has been less thorough than my study of the roots.

Evidence of a different sort, though somewhat indirect, may be cited. At different times Rayner has reported isolating *Phoma radicis* from immature fruits as well as from seeds of *Calluna;* in fact, judging from a careful perusal of her publications, her isolations have invariably come from above-ground plant parts; but when working with the cranberry (*21*), to her surprise she failed to find the fungus in seed cultures, a result, incidentally, confirmed by the writer (p. 819). The United States Department of Agriculture has conducted investigations of cranberry diseases continuously for 30 years, in the course of which cultures have been made from more than 100,000 individual berries, including a considerable number that were sound and immature, as reported by Shear (*28, 29, 30*); Shear, Stevens, and Bain (*31*); Rudolph and Franklin (*27*); Stevens (*33*); Stevens and Bain (*34, 35, 36*); Bain (*3*); and Bergman and Wilcox (*5*). *Phoma radicis* has not appeared once in all these cultures, a circumstance that appears incredible if the fungus is invariably present in the fruit tissues and grows as readily on ordinary culture media as Rayner's publications indicate.

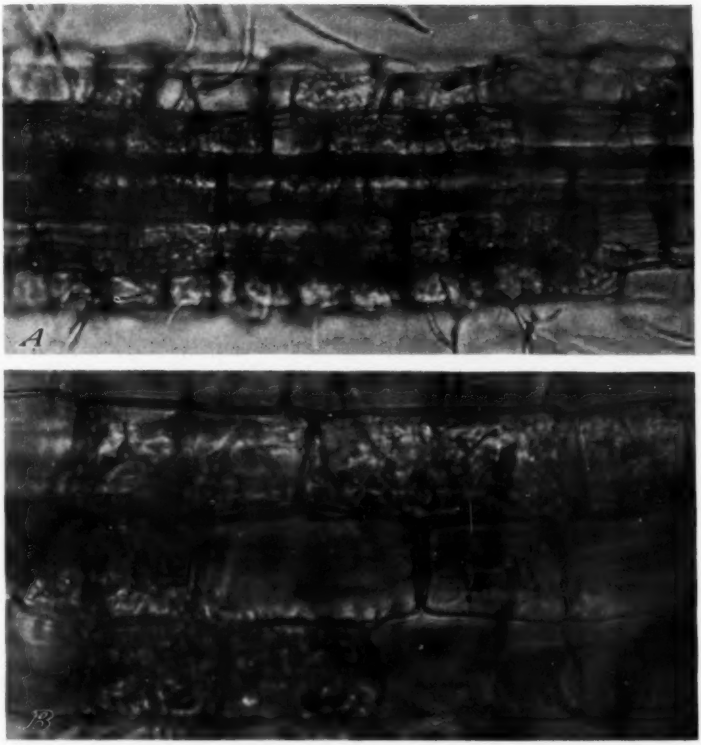
It appears to the writer that the weight of evidence is distinctly against either *Phoma radicis* or true mycorhizal fungi occurring systemically in the cranberry.

(3) Universal infection would be inevitable only in case systemic infection were invariable. In the writer's opinion, heath plants in general are mycorhizal because humus-inhabiting fungi of different species, capable of entering into the relation, are widely disseminated.

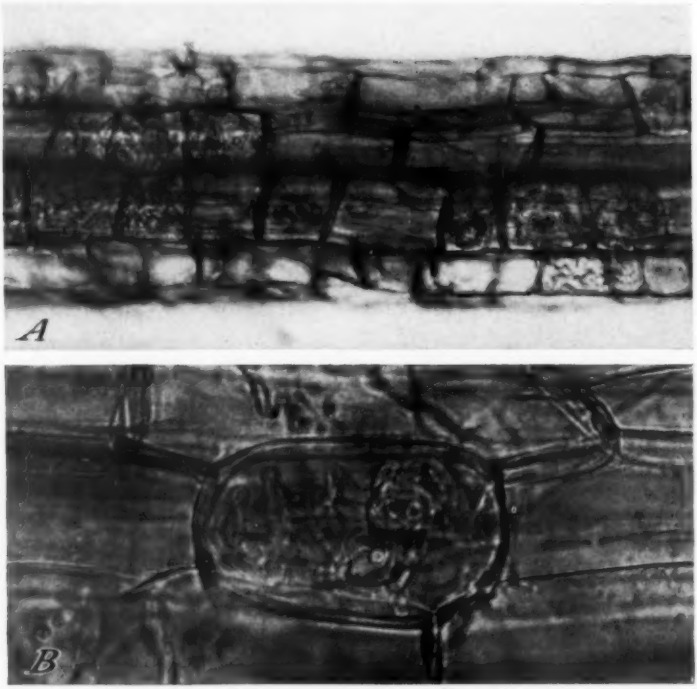
(4) The assumed impossibility of growing seedlings free from systemic infection is also based on universal invasion of seeds. However, mycorhizal infection, applying the term in its ordinary sense to the union of fungus and root (the hyphal complex relation in the case of heath plants), is quite another matter, and heath plants may be grown entirely free from the fungus or fungi involved, as has been demonstrated by the writer and by Stahl (*32*), Christoph (*6*), Knudson (*13, 14*), and Friesleben (*9, 10, 11*).

(5) No proof has yet been advanced that *Phoma radicis* assumes two different forms in different parts of the plant. The intracellular hyphal complex structures in roots, which Rayner attributes to *Phoma*, are produced by other fungi in *Calluna* (*6*), *Vaccinium corymbosum* and *V. pennsylvanicum* (*7*), *V. myrtillus*, *V. vitis-idaea*, and *V. uliginosum* (*10*), and *V. macrocarpon*.

(6) The hypothesis of obligate relationship in the heaths was advanced to explain certain definite results obtained in a series of experiments, and its validity depends upon how completely and exclusively it meets observed conditions in its own and related fields. In the experiments on which it was founded (*17*) plants grown in agar cultures from "adequately" sterilized seed of *Calluna* failed to produce roots until inoculated with a fungus derived from the fruit (or seed?) of the plant. Examination of the plants disclosed systemic infection by extremely attenuated hyphae in the inoculated series but no infection in the other lot. The fungus was therefore considered to be the stimulative factor bringing about root formation. To further strengthen the hypothesis, numerous cases of natural systemic infection in the same and related plants have been described subsequently, and in the genus *Vaccinium* (*21*) a similar series of agar plantings was made. Plants from seed sterilized in a manner which would have prevented root formation in *Calluna* here produced roots; but systemic infection




Synthetic mycorhiza in water-agar culture of cranberry, produced by *Ledum* endophyte: *A*, Distribution of infected cells, × 375; *B*, detailed structure, × 750.




Natural mycorhiza in cranberry: *A*, Distribution of infected cells, × 375; *B*, detailed structure, × 1,250.

was also present, the fungus having come from observed mycelium of the same type in the endosperm of resting seed. The coincidental development of roots with infection in these plants was cited as corroborative evidence of the obligate relationship.

Opposing this interpretation, Knudson (*13*, *14*) and Friesleben (*9*, *10*, *11*) attributed root suppression in sterilized media directly to an inhibitory factor existing in the substratum. In justification of their stand they have shown that if a given lot of sterilized seed is planted on two substrates differing in specified chemical or biochemical make-up, root growth is suppressed on one medium but not on the other. Such results would not be expected according to Rayner's hypothesis of root stimulation, because (1) if the sterilization were severe enough to completely eliminate seed-inhabiting fungi, roots should not develop in either lot, while (2) if the mycelium were not eliminated, both lots should produce roots. In the writer's opinion, the evidence for the cranberry is less convincing in this respect because the rooting capacity of the cranberry is too great to permit complete root suppression as long as any part of the stem is alive; but the roots are nevertheless extremely sensitive to the composition of the substratum.

The other phase of the obligate hypothesis, namely, actual existence of systemic infection, has been discussed above. Until the invariable existence of systemic infection is more conclusively demonstrated, and until there is more definite proof that root suppression is due solely to lack of such infection, the hypothesis of obligate root stimulation by fungi in the heath family cannot be considered valid.

## SUMMARY

Mycorhizal fungi were isolated from "hyphal complex" cells in roots of four ericaceous species—*Vaccinium macrocarpon*, *V. canadense*, *Chamaedaphne calyculata*, and *Ledum groenlandicum*—a distinct type from each species.

The four fungi have not fruited in culture and hence could not be identified. They were obviously specifically distinct from each other and from *Phoma radicis*.

All four fungi produced the hyphal complex form of mycorhiza in cranberry seedlings grown in sterilized agar. The cranberry fungus also produced mycorhiza in cranberry seedlings when the latter were grown in an inoculated artificial soil composed of ground cork and sand. The hyphal complex form of mycorhiza was completely lacking when the plant cultures were not inoculated with the endophytes.

There was no evidence of injury due to parasitism after the seedlings were inoculated with the fungi.

In agar cultures the cranberry fungus entered into the mycorhizal relation much more freely if carbohydrates were withheld from the medium.

Seedlings grown in nutrient-free distilled-water agar derived no measurable benefit from the presence of mycorhiza.

Cranberry roots were extremely sensitive to the composition of the substrate. Their growth was more or less suppressed on nearly all nutrient agars tried.

Some nutrient agars were rendered still more toxic to roots when the endophytes were introduced into the media; others were made

decidedly less toxic. These changes in the media were not correlated with mycorhizal infection.

Systemic infection of the type attributed to *Phoma radicis* by some investigators could not be found either in the seedlings or in prepared slides from field-grown material.

The hypothesis of systemic infection by mycorhizal fungi and of its obligate relationship to root formation in the heath family is examined critically. It is shown that the hypothesis fails to conform to observed facts in some important respects.

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# THE LOSS OF CAROTENE IN HAYS AND ALFALFA MEAL DURING STORAGE[1]

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## INTRODUCTION

For several years it has been recognized that the amount of carotene in plant materials is a measure of their vitamin A potency. This has been demonstrated best probably in the case of alfalfa hay (*7*).[3] It is also known that carotene is frequently deficient in farm rations (*6*, *14*, *15*, *16*). From an economic standpoint, therefore, it is important to know the conditions that influence the carotene content of farm feeds, especially of such feeds as alfalfa, timothy, and clover hays, which frequently furnish very nearly all the carotene in dairy rations, and which may, or may not, be rich sources of this factor (*20*).

This paper presents the results of experimental work conducted to determine the rate of loss of carotene in alfalfa and timothy hays and in alfalfa meals during storage under practical conditions at different seasons of the year. As the natural green color, which is used as a factor in the grading of market hays, is in general a most useful index of carotene content (*20*), the change in color that occurred during the storage of some of these hays was noted, and the results are included in this paper.

Considerable work has been done on the oxidative decomposition of pure carotene under various conditions. It is unnecessary to review this work here except to say that, although a number of oxidation products with vitamin A activity have been artificially prepared from carotene (*4*, *12*, *13*), the only naturally formed decomposition products that seem to have been tested for biological activity were tested in this laboratory and found to be inactive. There has, however, been work in other laboratories on the losses of carotene in the making and preserving of hays and meals by various methods; a review of this work may throw some light on the conditions that affect these losses.

## REVIEW OF OTHER INVESTIGATIONS

Russell, Taylor, and Chichester (*19*) showed that carotene disappears rapidly from freshly cut plant materials. They observed a loss of 80 percent of the carotene in alfalfa during the first 24 hours of drying in the field. Similar losses have been noted by Hauge and Aitkenhead (*9*) and by Guilbert (*5*) and others when alfalfa was dried in the sun. Work with alfalfa, reported by this laboratory (*20*),

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[3] Reference is made by number (italic) to Literature Cited, p. 846.

shows that market hays frequently contain only one-tenth to one-fifth as much carotene as is ordinarily found in the growing plant.

A number of investigators have compared various methods of treating alfalfa to determine the conditions that affect the destruction of carotene. Russell (*18*), in his earlier work, found that leaves from alfalfa hay that had been quickly dried in a tunnel drier at 127° to 129° C., had more than seven times the vitamin A potency of leaves from a "poorly cured," "brownish green" hay that had been dried by exposure for several days in the field. In later experiments he and his coworkers (*19*) found that in two freshly cut samples of alfalfa dried either in a tunnel drier or in a rotary machine in which the material was momentarily exposed to a much higher temperature (650° to 750° C.), the carotene content per unit of dry matter was as great as that of freshly cut material, and 2 to 10 times as great as that of samples from the same hay that had been dried in the field. The loss of carotene in the field-dried hay was particularly rapid during daylight.

In the work of Hauge and Aitkenhead (*9*), and in later experiments by Hauge (*8*), it was found that, if freshly cut alfalfa was first autoclaved, there was no destruction of carotene when it was dried in the sun. Hauge and Aitkenhead also found that the autoclaved material did not lose carotene when incubated subsequently; whereas, when unautoclaved material was incubated, it lost carotene at a rate which increased as the temperature of incubation was increased from 4° to 37° C. They attributed the loss of carotene during the drying of alfalfa in sunlight to the action of enzymes the activity of which was increased by the higher temperatures produced by the sun's rays.

Guilbert (*5*) also found evidence of the presence of enzymes of this nature in alfalfa, but his results differ in several respects from those of Hauge and Aitkenhead. His analytical procedure, although it differed somewhat from theirs, would be expected to give similar results. Guilbert found that samples of alfalfa leaves that had been artificially dried by methods that should have destroyed any enzymes present, still lost carotene at a rapid rate when exposed to sunlight, and lost a considerable quantity even when standing in the dark. Alfalfa leaves that had been dried in vacuo at 100° C. lost 6.8 percent of their carotene when allowed to stand in the dark for 12 hours at 65°; and other leaves, similarly dried, lost nearly 10 times this amount when exposed to sunlight for the same length of time at the same temperature. Autoclaved samples of alfalfa lost 26.5 to 53.8 percent of their carotene when subsequently dried in sunlight, whereas unautoclaved samples that were directly dried in sunlight lost 46.6 to 69.5 percent.

In work with alfalfa meals, Guilbert also reports an artificially dehydrated sample which lost 83.3 percent of its carotene when exposed to sunlight for 40 hours. He compares the keeping qualities of the carotene in meals made from sun-dried and artificially-dried samples from each of three lots of alfalfa. In one lot the method of drying had no effect; in the other two, the losses of carotene were 50 percent greater in the artificially dried samples. The losses of carotene in these meals varied with the temperature at which they were stored. In a meal stored at 0° to 5° C., no loss was detectable in 8 weeks; in one stored at 20° to 30° about 30 percent was lost in that

time. Guilbert also determined the loss of carotene with three sun-dried samples of hay that were stored in the dark. They lost 9 percent of their carotene from November to April, and 30 percent from April to August.

The results recorded in the present paper have been presented in part before various groups, and abstracts of these reports have been published (*10*, *20*, *24*). In connection with various feeding experiments in this laboratory, a considerable number of market hays have been analyzed for carotene. Whenever in a lot, or purchase, of hay a bale has been found in which the hay showed evidence of fermentation such as a "caked" or "set" condition, or a "ground," "tobacco," or "sweated" odor, the carotene content has been decidedly lower than in bales in which this condition did not exist (*20*). As is well known, this spoilage, which is the result of bacterial action, is due to excessive moisture in the hay during storage. Woodward has studied this problem (*25*).

The fineness to which a hay is ground may also affect the rate of loss of carotene both in the preparation of meals and during subsequent storage. In grinding hays to a fine powder in a ball mill, a considerable part (average about 25 percent) of the carotene is lost, and the powdered material loses carotene very rapidly during subsequent storage (*23*). This does not occur, however, when the hays are ground in a Wiley mill equipped with a sieve with holes 0.5 mm in diameter.

From the foregoing statements it is evident that the destruction of carotene may proceed in hays in which all enzymes presumably have been destroyed. Loss of carotene occurs even when these hays are in the dark, and is accelerated greatly by exposure to sunlight, by an increase in the temperature at which they are allowed to stand, and by conditions favoring bacterial action in the hay. In considering the factors that influence the rate of destruction of carotene in hays, it must be borne in mind that pure carotene itself is oxidized on standing in air (*22*), that the process is much more rapid in sunlight, at higher temperatures, and generally when impurities are present (*2*, *3*). Evidence exists that the destruction of carotene in such preparations may be autocatalytic (*22*), or may be influenced by a number of catalysts (*3*) and anticatalysts or antioxidants (*1*, *3*, *21*). The process in hays, therefore, would be expected to be quite complicated and possibly quite variable.

## EXPERIMENTS IN STORAGE OF HAYS

The hays used in this experiment were stored in bales in a dark, unheated barn loft at Beltsville, Md., and analyzed from time to time for carotene according to the usual procedure in this laboratory (*17*, *pp. 461–462*). The maximum and minimum outside daily temperatures were read and their average was taken as the "mean daily temperature." These mean daily temperatures were averaged to obtain the mean temperature for the periods of storage. The rate of loss of carotene in percentage per month was calculated by means of the equation $A(100-x)m=B$, in which $x$ represents the rate, $A$ and $B$ are the initial and final carotene figures, respectively, for the period of storage, and $m$ is the length of the period in months.

## LOSS OF CAROTENE DURING STORAGE AT VARIOUS TEMPERATURES

### ALFALFA HAY

The results of the experiment with alfalfa hay are shown in table 1, in which the percentage rates of loss of carotene per month are arranged in three groups according to the mean temperature during the period of storage. These three groups include samples stored at the following mean temperatures: (A) At 7.2° C. or less, (B) at 7.2°+ to 18.9°, and (C) at above 18.9°. Group A represents the temperatures that prevail at Beltsville, Md., during December, January, February, and frequently in March and November; group C the temperatures during June, July, August, and to a considerable extent in September; and group B the usual temperatures during the fall and spring months.

TABLE 1.—*Carotene content of alfalfa hays at different times during storage, percentage loss of carotene per month, and effect of temperature on this loss*

| Samples of hay [1] (laboratory designation) | Date of analysis | Carotene content | Mean temperature since preceding analysis | Loss of carotene per month at temperatures indicated [2] | | |
|---|---|---|---|---|---|---|
| | | | | A, 7.2° C. or less | B, 7.2°+ to 18.9° C. | C, above 18.9° C. |
| | | *Milligrams per kilogram* | *° C.* | *Percent* | *Percent* | *Percent* |
| Alfalfa hay, U. S. No. 1: | | | | | | |
| | Mar. 25, 1933 | 37.1 | | | | |
| | May 19, 1933 | 34.5 | 12.2 | | 3.9 | |
| Lot 50, bale 1 | June 8, 1933 | 31.4 | 19.4 | | | 13.8 |
| | July 21, 1933 | 22.5 | 20.6 | | | 20.8 |
| | Aug. 19, 1933 | 16.0 | 24.4 | | | 29.8 |
| | Nov. 16, 1933 | 121.0 | | | | |
| | July 25, 1934 | 52.1 | 9.4 | | 8.0 | |
| T-barn, bales 101, 102 | Nov. 19, 1934 | 46.2 | 16.1 | | 3.1 | |
| | Apr. 8, 1935 | [3] 33.4 | 3.9 | 6.7 | | |
| | June 18, 1935 | 29.8 | 15.6 | | 4.8 | |
| | Aug. 29, 1935 | 22.9 | 23.3 | | | 10.5 |
| | Jan. 4, 1934 | 33.4 | | | | |
| | Feb. 14, 1934 | 32.2 | −0.6 | 2.6 | | |
| C-barn, bale 110 | July 26, 1934 | 21.0 | 13.9 | | 7.6 | |
| | Nov. 19, 1934 | 19.0 | 16.1 | | 2.6 | |
| | Apr. 6, 1935 | 16.3 | 3.9 | 3.3 | | |
| | June 15, 1935 | 13.2 | 14.4 | | 8.8 | |
| | Jan. 20, 1934 | 30.5 | | | | |
| | July 16, 1934 | 16.7 | 12.2 | | 9.8 | |
| Lot 57, bales 111, 112 | Nov. 20, 1934 | 13.9 | 16.7 | | 4.3 | |
| | Apr. 9, 1935 | [3] 12.5 | 3.9 | 2.3 | | |
| | June 14, 1935 | [3] 10.6 | 15.0 | | 7.3 | |
| | Mar. 22, 1934 | 38.0 | | | | |
| | July 5, 1934 | 25.2 | 16.1 | | 11.2 | |
| Lot 58, bales 117, 118 | Nov. 20, 1934 | [4] 15.8 | 17.8 | | 9.9 | |
| | Mar. 29, 1935 | 16.0 | 3.9 | −1.0 | | |
| | June 12, 1935 | 14.4 | 13.9 | | 4.2 | |
| | Aug. 27, 1934 | 28.2 | | | | |
| | Jan. 24, 1935 | 21.1 | 9.4 | | 5.8 | |
| Lot 59, bales 1, 2 | Mar. 29, 1935 | 20.2 | 3.9 | 2.0 | | |
| | June 22, 1935 | 17.5 | 15.0 | | 5.0 | |
| | Aug. 30, 1935 | 13.0 | 23.3 | | | 12.5 |
| | Oct. 3, 1934 | 37.8 | | | | |
| | Jan. 18, 1935 | 29.5 | 7.2 | 6.8 | | |
| Lot 59, bale 3 | Mar. 29, 1935 | 27.2 | 3.9 | 3.4 | | |
| | June 13, 1935 | 25.5 | 13.9 | | 2.5 | |
| | Aug. 30, 1935 | 19.1 | 23.3 | | | 10.5 |

[1] Hays graded by representatives of the Division of Hay, Feed, and Seed, Bureau of Agricultural Economics. Each "lot" of hay is a separate purchase.

[2] Data are arranged in 3 groups according to the temperature ranges indicated and depending upon the mean temperature at which the sample was stored during the period since the preceding analysis. Average temperature for 1933–35 at Beltsville, Md.: For winter months (December, January, February), 0.9° C.; for cool fall and spring months, 8.3°; for warm fall and spring months, 12.2°; and for summer months (June, July, August), 22.4°.

[3] 1 bale during this period was stored in the attic of the laboratory. This attic was unheated, but probably was warmer than the barn loft during the winter.

[4] 1 of triplicate determinations omitted; probably some destruction of carotene in it.

TABLE 1.—*Carotene content of alfalfa hays at different times during storage, percentage loss of carotene per month, and effect of temperature on this loss*—Continued

| Samples of hay (laboratory designation) | Date of analysis | Carotene content | Mean temperature since preceding analysis | Loss of carotene per month at temperatures indicated | | |
|---|---|---|---|---|---|---|
| | | | | A, 7.2° C. or less | B, 7.2°+ to 18.9° C. | C, above 18.9° C. |
| | | *Milligrams per kilogram* | ° *C.* | *Percent* | *Percent* | *Percent* |
| Alfalfa hay, U. S. No. 1—Continued. | | | | | | |
| | Nov. 5, 1934 | 43.4 | | | | |
| | Feb. 13, 1935 | 38.5 | 2.8 | [3] 3.6 | | |
| Lot 64, 10 bales [2] | Apr. 15, 1935 | 34.0 | 7.2 | [3] 5.8 | | |
| | June 21, 1935 | 29.3 | 16.7 | | [3] 6.5 | |
| | Sept. 9, 1935 | 17.2 | 23.3 | | | 18.1 |
| | Jan. 14, 1935 | 41.3 | | | | |
| | Mar. 30, 1935 | 39.0 | 3.3 | 2.2 | | |
| H. O., bale 1 | June 21, 1935 | 28.3 | 15.0 | | 10.9 | |
| | Aug. 30, 1935 | 16.5 | 23.3 | | | 20.9 |
| | Jan. 15, 1935 | 81.8 | | | | |
| | Mar. 30, 1935 | 72.5 | 3.3 | 4.7 | | |
| H. O., bale 1a | June 21, 1935 | 54.8 | 15.0 | | 9.6 | |
| | Aug. 29, 1935 | 30.0 | 23.3 | | | 23.3 |
| Alfalfa hay, U. S. No. 2: | | | | | | |
| | Jan. 31, 1935 | 14.1 | | | | |
| H. O., bale 2 | Apr. 5, 1935 | 14.6 | 5.6 | −1.0 | | |
| | Jan. 31, 1935 | 12.4 | | | | |
| H. O., bale 2a | Mar. 30, 1935 | 12.7 | 5.6 | −1.0 | | |
| | Feb. 2, 1934 | 11.4 | | | | |
| | July 26, 1934 | 7.6 | 12.2 | | 6.8 | |
| Lot 54, bales 114, 115 | Nov. 20, 1934 | 5.4 | 16.1 | | 8.6 | |
| | Apr. 16, 1935 | [3] 4.7 | 3.9 | 2.8 | | |
| Alfalfa hay, U. S. No. 3: | | | | | | |
| | Aug. 28, 1934 | 7.0 | | | | |
| Lot 61, bales 1, 2 | Apr. 19, 1935 | 5.3 | 7.8 | | 3.5 | |
| | Sept. 26, 1934 | 8.7 | | | | |
| Lot 61, bale 3 | Apr. 5, 1935 | 9.5 | 6.1 | −1.0 | | |
| Average | | | | 2.6 | 6.6 | 17.8 |

[3] 1 bale during this period was stored in the attic of the laboratory. This attic was unheated, but probably was warmer than the barn loft during the winter.
[2] Same 10 bales used throughout except on Nov. 5, 1934.

It will be noted that the rate of loss of carotene during storage varied greatly with the different hays in each group shown in table 1, and that the average percentage loss per month with each group varied with the temperature at which the hays were stored. The rate of change in carotene content per month with the hays in group A varied from an increase of 1 percent to a loss of 6.8 percent; in group B, the loss varied from 2.5 to 11.2 percent; and in group C, from 10.5 to 29.8 percent. The average losses of carotene per month in these hays were, respectively, for group A, 2.6 percent; for group B, 6.6 percent; and for group C, 17.8 percent. Of the hays in group C, the average rate of loss of carotene for those that were stored the first summer after cutting was 21.1 percent per month, and that for the few samples (T-barn, bales 101 and 102; lot 59, bales 1 and 2; lot 59, bale 3) in storage the second summer after cutting was 11.2 percent per month.

These data for the loss of carotene during the storage of hays in the summer months indicate that the rate of loss of carotene under conditions otherwise comparable may possibly decrease with the age of the hay; but the errors and variations in determining such data are too great to permit a clear demonstration of this fact. A loss of carotene of 17.4 percent between November 16 and November 30, 1933, was observed with the hay marked T-barn, bales 101 and 102. This suggests that carotene may be lost at a very rapid rate in sun-

cured hays for some time after they are dried and baled. In none of the bales used in this experiment was there any evidence of fermentation or spoilage. The differences in the rate of loss of carotene with different hays at the same average temperature may actually be due in part to differences in the keeping qualities of the hays, but it must be borne in mind that the figures are derived from differences in determinations that are themselves subject to considerable error, and that samples stored at a given average temperature actually were exposed at times to quite different conditions.

### Timothy Hay

The data obtained on the loss of carotene in timothy hay during storage are shown in table 2. It will be noted that the rate of decomposition of carotene during the storage of timothy hay is about the same as with alfalfa, and is similarly affected by variations in temperature. In 11 lots of timothy hay stored at temperatures between 6.0°+ and 18° C. the average loss of carotene was 6.3 percent per month; in 24 samples of alfalfa hay similarly stored the loss was 6.6 percent per month.

Table 2.—*Carotene content of timothy hays at different times during storage, percentage loss of carotene per month, and effect of temperature on this loss*

| Samples of hay (laboratory designation) and date of analysis [1] | Carotene content | Mean temperature since preceding analysis | Loss of carotene per month at temperatures [2] indicated [3] | |
|---|---|---|---|---|
| | | | 7.2° C. or less | 7.2°+ to 17.8° C. |
| | *Milligrams per kilogram* | *° C.* | *Percent* | *Percent* |
| Lot 43, bales 107, 108: | | | | |
| Mar. 1, 1934 | 19.9 | | | |
| July 16, 1934 | 13.9 | 15.0 | | 7.7 |
| Lot 44, bales 1, 2: | | | | |
| Aug. 28, 1934 | 9.2 | | | |
| Jan. 3, 1935 | 6.8 | 10.6 | | 6.9 |
| May 1, 1935 | 6.3 | 5.0 | 1.9 | |
| Lot 44, bales 115, 116: | | | | |
| June 4, 1934 | 8.6 | | | |
| Feb. 14, 1935 | 6.2 | 12.8 | | 3.8 |
| May 2, 1935 | 5.2 | 8.3 | | 6.5 |
| Lot 46, bales, 1, 2, 3, 4, 5: | | | | |
| Dec. 11, 1934 | 23.6 | | | |
| May 11, 1935 | 18.8 | 5.6 | 4.4 | |
| Dec. 2, 1935 | 11.7 | 17.8 | | 6.8 |
| Lot 46, bale 6: | | | | |
| Dec. 11, 1934 | 18.4 | | | |
| Jan. 25, 1935 | 18.4 | 2.2 | .0 | |
| Apr. 25, 1935 | 17.0 | 5.6 | 2.6 | |
| Dec. 2, 1935 | 11.7 | 17.8 | | 5.0 |
| Lot 48, bales, 1, 2, 3, 4, 5: | | | | |
| Jan. 18, 1935 | 10.7 | | | |
| Apr. 18, 1935 | 9.6 | 4.4 | 3.5 | |
| Dec. 4, 1935 | 7.0 | 17.2 | | 4.0 |
| Lot 48, bale 6: | | | | |
| Jan. 17, 1935 | 10.3 | | | |
| Apr. 18, 1935 | 6.7 | 4.4 | 13.2 | |
| Dec. 5, 1935 | 6.4 | 17.2 | | .6 |
| Lot 39, bales 101, 102: | | | | |
| Jan. 6, 1934 | 9.2 | | | |
| July 16, 1934 | 4.5 | 10.0 | | 10.7 |
| Lot 42, bales 104, 105: | | | | |
| Jan. 6, 1934 | 9.0 | | | |
| July 5, 1934 | 4.7 | 9.4 | | 10.3 |
| Average | | | 4.3 | 6.2 |

[1] Each lot of hay is a separate purchase.

[2] Average temperature for 1933–35 at Beltsville, Md.: For winter months (December, January, February) 0.9° C.; for cool fall and spring months, 8.3°; for warm fall and spring months, 12.2°; and for summer months (June, July, August), 22.4°.

[3] Results arranged in 2 groups according to mean temperature during periods between analyses.

### Clover Hay

One lot of clover hay was included in the experiment. On June 24, 1934, it contained 16.8 mg of carotene per kilogram of dry matter. On January 2, 1935, and April 15, 1935, the carotene determinations each gave 8.8 mg per kilogram. The average temperature for the total period of storage was 11.1° C., and the average percentage loss of carotene per month was 6.3 percent. In six lots of alfalfa that were stored at temperatures ranging from 9.4° to 12.8° C., the average loss of carotene per month was 6.6 percent.

### RELATION OF GREEN COLOR IN HAY TO CAROTENE CONTENT

The writers have compared the decrease in green color during storage, as determined by hay specialists of the Bureau of Agricultural Economics, with the loss of carotene during the storage of several lots of alfalfa hay. In one lot there was a decrease in green color from 71 percent to 56 percent—a loss of 21 percent of the natural color originally present. The corresponding loss in carotene content was from 32 to 13 mg per kilogram, or nearly 60 percent. With another hay a decrease in color from 69 to 54 percent—a loss of 22 percent—was accompanied by a drop in the carotene content from 38 to 14 mg per kilogram, or somewhat over 60 percent. A third lot of hay, which changed from 55 percent color to 44 percent—a loss of 20 percent—lost 70 percent of its carotene content. Apparently the percentage loss of carotene is much greater than the corresponding percentage loss of natural green color—about three times as great with the samples just cited.

## EXPERIMENT IN STORAGE OF MEALS

For the study of the loss of carotene during the storage of meals made from alfalfa hay, several bales were taken from each of four lots, or purchases, of hay. The bales selected from each lot were similar in color and leafiness. One bale from each lot was graded by hay specialists of the Bureau of Agricultural Economics, and different portions of it were ground by them in a hammer mill to different degrees of fineness, so that the particles passed through ⅛-, ¼-, or ¾-inch circular mesh screens during grinding.

The color and the carotene content of these meals were then determined, and the determinations were repeated from time to time during storage from about April 1, 1934, to October 1, 1935. Samples of the meals were stored in individual bags in a dark, unheated part of a building in Washington, D. C. This place was somewhat more sheltered from the effect of outdoor weather changes than the barn loft referred to above. The other bales from each lot of hay were stored in this loft and analyzed from time to time. The outdoor temperatures were read at Beltsville (near Washington, D. C.); and the average temperatures for the periods of storage of the meals and the rates of loss of carotene during storage were calculated in the same manner as for the hay experiment.

### DETERMINATIONS OF CAROTENE CONTENT AND COLOR

The results of the analyses for the carotene content of the meals, as determined from time to time during storage, are given in table 3.

TABLE 3.—*Losses of carotene and green color during the storage of meals made from alfalfa hay*

| Meal prepared from bale no.— | Carotene content | | | | | Retention of natural green color | | | | | Green color of the hay on Apr. 7, 1924 [1] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Date of analysis | Meal ground to pass— | | | Average | Date observed | Meal ground to pass— | | | Average | |
| | | ⅛-inch mesh | ¼-inch mesh | ¾-inch mesh | | | ⅛-inch mesh | ¼-inch mesh | ¾-inch mesh | | |
| | | *Milligrams per kilogram* | *Milligrams per kilogram* | *Milligrams per kilogram* | *Milligrams per kilogram* | | *Percent* | *Percent* | *Percent* | *Percent* | *Percent* |
| 116 | Apr. 23, 1934 | 12.8 | 13.5 | 12.5 | 12.9 | Apr. 11, 1934 | 35 | 33 | 30 | 33 | 57 |
| | Dec. 15, 1934 | 8.2 | 8.8 | 7.5 | 8.2 | Nov. 20, 1934 | 34 | 33 | 29 | 32 | |
| | May 21, 1935 | 6.1 | 7.8 | 5.4 | 6.4 | Apr. 5, 1935 | 28 | 26 | 25 | 26 | |
| | Oct. 14, 1935 | 5.0 | 5.0 | 5.0 | 5.0 | Oct. 2, 1935 | 32 | 26 | 20 | 26 | |
| 119 | Apr. 25, 1934 | 43.9 | 39.2 | 38.1 | 40.4 | Apr. 11, 1934 | 48 | 47 | 48 | 48 | 73 |
| | Nov. 30, 1934 | 29.4 | 25.5 | 21.8 | 25.6 | Nov. 20, 1934 | 39 | 39 | 33 | 37 | |
| | May 22, 1935 | 20.4 | 19.0 | [2] 18.3 | 19.2 | Apr. 5, 1935 | 33 | 36 | 31 | 33 | |
| | Sept. 4, 1935 | 14.3 | 14.8 | 12.6 | 13.9 | Oct. 2, 1935 | 35 | 34 | 24 | 31 | |
| 109 | Mar. 28 and Apr. 9, 1934 | 43.9 | 42.1 | [3] 45.3 | 43.8 | Apr. 11, 1934 | 60 | 54 | 53 | 56 | 71 |
| | Nov. 24 and Nov. 30, 1934 | 24.5 | 24.4 | 23.1 | 24.0 | Nov. 20, 1934 | 55 | 50 | 48 | 51 | |
| | May 21, 1935 | 21.1 | [2] 19.4 | [2] 21.1 | 20.5 | Apr. 5, 1935 | 43 | 40 | 41 | 41 | |
| | Oct. 12, 1935 | 13.6 | 13.6 | 13.9 | 13.7 | Oct. 2, 1935 | 45 | 41 | 39 | 42 | |
| 113 | Mar. 28, 1934 | 24.0 | 27.2 | 26.6 | 25.9 | Apr. 11, 1934 | 53 | 52 | 53 | 53 | 72 |
| | Nov. 27, 1934 | 16.3 | 18.7 | 18.3 | 17.8 | Nov. 20, 1934 | 42 | 42 | 42 | 42 | |
| | June 5, 1935 | 12.3 | 15.4 | 14.5 | 14.1 | Apr. 5, 1935 | 39 | 41 | 38 | 39 | |
| | Sept. 5, 1935 | [4] 9.9 | 10.1 | 9.0 | 9.7 | Oct. 2, 1935 | 39 | 33 | 40 | 37 | |

[1] The percentage of natural green color in the hay is not comparable with that in the meals.
[2] Determined May 13.
[3] Determined Apr. 9 and Apr. 16.
[4] Determined Sept. 4.

Table 4 shows the total percentage loss of carotene for the whole period of storage for each sample of meal.

TABLE 4.—*Total percentage loss of carotene and green color in alfalfa meals during period of storage (about Apr. 1, 1934, to Oct. 1, 1935)*

| Meal prepared from hay bale no.— | Loss of carotene in meal ground to pass— | | | Loss of green color in meals ground to pass— | | |
|---|---|---|---|---|---|---|
| | ⅛-inch mesh | ¼-inch mesh | ¾-inch mesh | ⅛-inch mesh | ¼-inch mesh | ¾-inch mesh |
| | *Percent* | *Percent* | *Percent* | *Percent* | *Percent* | *Percent* |
| 116 | 60.9 | 63.0 | 60.0 | 8.6 | 21.2 | 33.3 |
| 119 | 67.4 | 62.2 | 66.9 | 27.1 | 27.7 | 50.0 |
| 109 | 60.0 | 67.7 | 69.3 | 25.0 | 24.1 | 26.4 |
| 113 | 58.8 | 62.8 | 66.2 | 26.4 | 36.5 | 24.5 |
| Average | 64.0 | 63.9 | 65.6 | 21.8 | 27.3 | 33.5 |

In table 5 the rates of loss of carotene in the meals and those in the corresponding samples of baled hay are compared for different outdoor temperatures.

The percentage of natural green color retained in the meals is shown in table 3 along with the data for carotene content. The total percentage loss of green color as compared with that of carotene is shown in table 4.

TABLE 5.—*Comparison of the rates of loss of carotene in alfalfa meals and corresponding baled hays, stored at different average temperatures*

| Meal prepared from bale no.— | Loss of carotene in the meals[1] | | | | | | Loss of carotene in corresponding hays stored in bales | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Average outdoor temperature | Per month | Average outdoor temperature | Per month | Average outdoor temperature | Per month | Average outdoor temperature | Per month | Average outdoor temperature | Per month |
| | ° C. | *Percent* | °C. | *Percent* | ° C. | *Percent* | ° C. | *Percent* | ° C. | *Percent* |
| 116 | 6.1 | 4.6 | 16.7 | 5.7 | 20.6 | 5.0 | 3.9 | 2.8 | 16.1 | 8.6 |
| 119 | 5.6 | 4.1 | 17.8 | 6.3 | 22.2 | 8.7 | 3.9 | [2] +1.0 | 15.6 | 7.4 |
| 109 | 5.6 | 2.7 | 17.2 | 7.6 | 20.6 | 7.9 | 3.9 | 3.3 | 15.0 | 5.4 |
| 113 | 7.2 | 3.6 | 17.2 | 4.6 | 22.8 | 11.9 | 3.9 | 2.3 | 15.6 | 5.8 |
| Average | 6.1 | 3.8 | 17.2 | 6.0 | 21.6 | 8.4 | 3.9 | 1.8 | 15.6 | 6.8 |

[1] Each datum under percent per month is the average for all grindings from each bale for the temperature indicated.
[2] With this hay there was an apparent gain of 1 percent per month instead of a loss during storage.

From the data in tables 3, 4, and 5, the following conclusions appear justified:

(1) The rate of loss of carotene in the meals was not affected by the fineness to which they were ground. For the total period of storage, the meals ground to pass ⅛-, ¼-, and ¾-inch meshes lost on an average 64.0, 63.9, and 65.6 percent, respectively.

(2) The percentage loss of color in the meals was unquestionably much smaller than that of carotene. The changes in color, however (table 4), are quite confusing. True, the meals faded during storage, and on an average the loss of green color was greater in the more coarsely ground meals, but the individual results are inconsistent.

(3) When due allowance is made for the errors in the data, it is evident that the rate of loss of carotene in the meals during storage was quite similar to that in the corresponding hays, and that in both cases these losses were similarly affected by the temperature at which the samples were stored.

In considering the loss of carotene in the meals, the question arises as to the loss that may have occurred during the grinding of the meals. A comparison of the results for the hays with those for the meals would have no bearing on this question because in making the determination for carotene, all hays and meals were finally ground to the same degree of fineness. The finest meals, however, were about six times as coarse as the material used for analysis, which was ground to pass through the finest mesh screen of the Wiley mill. Unpublished data indicate that grinding in this way does not destroy the carotene. It would seem, therefore, that the grinding of the meals stored in this experiment may likewise have been accomplished without a considerable loss of carotene.

## SUMMARY

Data on the rate of loss of carotene and of color in hays and alfalfa meals during storage are presented.

It appears that on an average the rates of loss of carotene in alfalfa, timothy, and clover hays under comparable conditions are quite simi-

lar. Therefore, the results for the different hays, taken together, may be summarized as follows:

Twenty-two samples of baled hays that were stored in a rather dark, unheated barn loft when the outside temperature was 7.2° C. or less (as is the case in December, January, February, and frequently in March and November, at Beltsville, Md., lost on an average about 3 percent of their carotene content per month.

With 34 samples of hay, which were similarly stored when the average outdoor temperatures ranged from 7.2°+ to 18.9° C. (as in April, May, October, and possibly September, at Beltsville), the average loss of carotene was 6.5 percent per month.

At outside temperatures above 18.9° C., the loss of carotene during storage increased greatly. For six samples of hay that were stored during the first summer after cutting, the loss was 21 percent per month, and for three samples stored further for a second summer, it was about 11 percent per month.

Meals from alfalfa ground to pass ⅛-, ¼-, and ¾-inch mesh screens lost carotene at the same rate during storage regardless of the degree of fineness to which they were ground, and at practically the same rate as the corresponding baled samples of hay.

With hays and meals, stored as above described, the percentage rate of loss of carotene was much more rapid than that of their natural green color. This fact must be taken into consideration in judging the carotene content or vitamin A potency of a hay from its color.

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# A STATISTICAL STUDY OF SAMPLING IN FIELD SURVEYS OF THE FALL POPULATION OF THE EUROPEAN CORN BORER[1]

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## INTRODUCTION

Surveys of insect abundance constitute an important part of the activities of economic entomologists. Such surveys not only provide fundamental information about insect pests on which control activities may be based but also may be used in studying the ecological relationships of these pests under actual field conditions and may thus contribute to the development and improvement of control methods. Obviously the methods used in the surveys of different insects and for different purposes differ from one another, depending as they do on the biology of the insect species and the objectives of the surveys.

The qualitative biological aspects of insect surveys are usually given adequate consideration, but the quantitative aspects of the sampling variability of all units of area-wide surveys have seldom, if ever, been analyzed under actual field conditions. For the analysis of the quantitative aspects of the sampling variability of such area-wide surveys of insect populations, the methods of analysis of the variance developed and introduced by Fisher (*4*)[3] are perfectly adapted. In the present paper these methods are applied to a study of sampling variability in the fall survey of the number of larvae per 100 cornstalks in the area infested by the one-generation brood of the European corn borer (*Pyrausta nubilalis* Hbn.)

## SURVEY METHODS AND DATA

Beginning with the year 1919, surveys to ascertain the abundance of the mature European corn borer larvae in the standing cornstalks in the one-generation area have been made each year by the Bureau of Entomology and Plant Quarantine, in cooperation with various State agencies. The data used in the present analysis cover 66 county units, part of which were surveyed in 1931 and part in 1932. For certain counties the data for both years, as separate units, were used, but the sets for the 2 years are not duplicates, some counties appearing 1 year but not the other. For both years counties located in Michigan, Ohio, Pennsylvania, and New York were included and the whole treated as one set of data. The detailed records are on file at the office of the United States Department of Agriculture laboratory for European corn borer research, Toledo, Ohio.

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[3] Reference is made by number (italic) to Literature Cited, p. 871.

As the infestation of feeding larvae in the one-generation area is confined entirely to the cornfields and almost entirely to the corn plants in the fields, the cornfield has constituted the basic unit of the surveys, and data were taken only with regard to the infestation in the corn plants.

The intensity of infestation was expressed as the number of borers per 100 plants and was estimated by determining the number of infested stalks out of 100 and the number of borers per infested stalk. The field was divided into quarters and from the approximate center of each quarter 25 consecutive plants were gathered. From the total number of infested stalks in the 25 stalk samples the average percentage of infested plants in the field or the number of infested plants per 100 was obtained. Then the infested stalks were examined in order to determine the average number of borers per stalk. In areas where the level of infestation in general was low, all the infested plants up to a maximum of 5 from the 100 plants were dissected. In areas where the level of infestation was higher, a maximum of 10 plants, if available, were dissected. The product of the number of infested plants in 100 and the average number of borers per infested plant gave the estimate of the number of borers per 100 plants in the field.

In conducting the surveys, a single county was almost invariably taken as the unit for which the sampling was carried out and the mean level of infestation reported. In general, the counties surveyed were fairly uniform in size and served very satisfactorily as basic areas for the analysis of the sampling variance. In the counties in which the infestation was expected to be relatively low, 25 fields were sampled, and in counties where the infestation was expected to be relatively higher, 20 fields were sampled. The fields were taken at random over the county and later combined into four groups, termed "sections", each group containing the same number of fields.[4]

The two main objectives kept in mind in the analysis of the data presented in this paper were (1) to determine the relative influence, on the reliability of the estimates of the field mean number of borers per 100 plants, of the variability (*a*) of the samples, taken in each field, from which the average percentage of infested stalks was computed and (*b*) of the samples from which the average number of borers per infested stalk was computed, and (2) to determine the amount of variability between fields within a county relevant to the reliability of the estimate of the county mean number of borers per 100 plants, and to compare it with the variability of samples within fields.

## ANALYSIS OF THE SAMPLING VARIABILITY OF THE ESTIMATES OF THE FIELD MEANS

### VARIANCE OF A PRODUCT DERIVED FROM THE VARIANCE OF THE FACTORS

As described, the sampling within fields was carried out by making two estimates in each field, one to determine the number of infested stalks per 100 and the other to determine the number of borers per infested stalk, the product of the two determinations giving an estimate of the average number of borers per 100 stalks. The chief problem in the analysis of the sampling within a field is, therefore, to assign to each factor the portion of the total sampling variability of the

[4] Where 25 fields were sampled, the thirteenth field was arbitrarily discarded.

estimate of the field mean which would be influenced by increasing or decreasing the number of samples in each set independently, and to express these portions in comparable terms.

This problem of analysis may be approached by the use of certain equations whereby the mean product, the variability of this product, and the variability of each factor of the product may be estimated. The mean product in terms of the constants of the factors may be estimated by the equation

$$\overline{X} = \overline{p}\,\overline{b} \qquad \text{Equation 1}$$

in which $\overline{X}$ is the estimated mean number of borers per 100 plants, $\overline{p}$ is the mean percentage of infestation, or the number of infested stalks per 100 stalks, and $\overline{b}$ is the mean number of borers per infested plant.

The variance of the product, also in terms of the constants of the factors, may be estimated by the equation

$$\sigma_{\overline{x}}^2 = \overline{p}^2\left(\frac{\sigma_b^2}{n'_b}\right) + \overline{b}^2\left(\frac{\sigma_p^2}{n'_p}\right) \qquad \text{Equation 2}$$

in which $p$ is the percentage of infested stalks out of the 25 stalk samples, $b$ is the number of borers in an infested stalk, $n'_p$ and $n'_b$ are, respectively, the number of infestation samples and the number of plants dissected, $\sigma_p$ and $\sigma_b$ are the standard deviations of the two factors, and $\sigma_p^2$ and $\sigma_b^2$ are the corresponding variances.

The variance of the percentage of infested stalks out of the 25 stalk samples may be estimated by the equation

$$\sigma_p^2 = \frac{\Sigma p^2 - \dfrac{(\Sigma p)^2}{n'_p}}{n_p} \qquad \text{Equation 3}$$

in which $n_p$ is the number of degrees of freedom available for estimating $\sigma_p^2$, or $n'_p - 1$.

The variance of the number of borers in an infested plant may be estimated by the equation

$$\sigma_b^2 = \frac{\Sigma b^2 - \dfrac{(\Sigma b)^2}{n'_b}}{n_b} \qquad \text{Equation 4}$$

in which $n_b$ is the number of degrees of freedom available for estimating $\sigma_b^2$, or $n'_b - 1$.

### A Comparison of the Variances of a Product Obtained by Two Methods

The variance of the field mean number of borers per 100 plants is underestimated when equation 2 is used, if a correlation exists between the number of infested stalks in the 25 stalk samples and the mean number of borers per infested plant in the corresponding quarters of the fields. Owing to the way the data were recorded in the fields, the correlation within fields could not be calculated for the years 1931 and 1932 when the data for this paper were obtained. But from data obtained in 1935 from 100 fields in Lenawee County, Mich.,

and the counties of Fulton, Hancock, Henry, Lucas, Ottawa, Putnam, and Sandusky, Ohio, the fact of a correlation between the two factors within fields was established and the effect this had on the standard error of the field mean number of borers per 100 plants was determined.

To show the existence of a correlation between the two factors just referred to, the number of infested plants out of 25 and the mean number of borers per infested plant were tabulated for the quarter of each of the 100 fields having the largest number of infested plants. Then the mean of the 100 samples was calculated for each factor. Similarly the data were tabulated and averaged separately for each of the other three quarters of each field. The mean numbers of infested plants out of 25 were 13.09, 10.53, 8.69, and 6.02 for the four quarters, while the mean numbers of borers per infested plant were, respectively, 1.75, 1.68, 1.55, and 1.39 for the same quarters. These data show that a decrease in the mean number of borers per infested plant is associated with a decrease in the number of infested plants.

The effect of this correlation on the standard error of the field mean number of borers per 100 plants was determined by completing two sets of calculations for each of the fields and comparing the mean results for groups of fields in the following manner: The first set of calculations was made to determine the mean number of borers per 100 plants for each field by equation 1 and to estimate the variance of the mean by equation 2. For the second set of calculations the product for each quarter of the field was obtained by multiplying the number of infested plants out of 25 by the mean number of borers per infested plant (2 or 3 plants were dissected in each quarter) and multiplying the result by 4 to obtain the mean number of borers per 100 plants for each quarter. The mean number of borers per 100 plants for each field was then obtained by averaging the results for the quarters of the fields. The variance of this general mean was estimated by computing the squared deviations of the product for each quarter from the mean product for the field and dividing by 4. The fields were then arranged in 5 groups, each of 20 fields, according to the field mean number of borers per 100 plants obtained from the first set of calculations. The group mean numbers of borers per 100 plants and the group mean standard errors are given in table 1 for each of the two methods of calculation.

TABLE 1.—*Range of estimates of the field mean number of European corn borers per 100 plants and the group mean standard errors derived by two methods of calculation, from 100 fields in northwestern Ohio and southeastern Michigan, 1935*

| First method | | Second method | |
|---|---|---|---|
| Mean | Standard error | Mean | Standard error |
| *Number* | *Number* | *Number* | *Number* |
| 15.1 | 5.25 | 15.7 | 5.63 |
| 32.5 | 9.65 | 33.2 | 10.41 |
| 60.0 | 18.09 | 61.5 | 18.03 |
| 81.1 | 24.36 | 84.1 | 25.04 |
| 151.2 | 41.21 | 156.9 | 43.21 |

It may be observed from table 1 that both the field mean number of borers per 100 plants of the groups of fields and the mean standard error are slightly underestimated by using equations 1 and 2, The average of the five groups shows that the standard error is underestimated about 3.7 percent. It appears, therefore, that on the average the standard error of the field mean number of borers per 100 plants may be estimated rather closely by using equations 1 and 2. On the basis of this comparison the validity of the results from the study described in this paper is assured if the 100 fields surveyed in 1935 may be taken as representative of the conditions existing in fields in general.

In order to assign to each of the two sets of samples taken in each field of the 1931 and 1932 surveys—one set to determine the number of infested stalks per 100 and the other to determine the number of borers per infested stalk—the portion of the total sampling variability of the estimates of the field mean numbers of borers per 100 plants which would be influenced by increasing or decreasing the number of samples in each set independently and to express these portions in comparable terms, the use of equations 1 and 2 is necessary. But these equations are incomplete because they make no allowance for any correlation which might exist between the two sets of samples. The complete form of the equations has been given by Reed (7).

The complete form of equation 1, adapted from Reed, is obtained by adding the term $r\sigma_p\sigma_b$ to the right-hand side of equation 1, and the complete form of equation 2 is obtained by adding the term $2r\overline{p}\overline{b}\sigma_{\bar{p}}\sigma_{\bar{b}}$ to the right-hand side of equation 2, in which $r$ is the coefficient of correlation between the percentage of infestation and mean number of borers per infested plant in the quarters of the field and $\sigma_{\bar{p}}$ and $\sigma_{\bar{b}}$ are the standard errors of the means of the two factors. It should be noted that in the complete form of the equations as applied to this problem there would be only four observations for both $p$ and $b$. Therefore $n'_p=n'_b=4$. The use of the complete form of equation 2 gives an estimate of the variance of the mean of the products from the quarters of the field.

### SAMPLING VARIABILITY WITHIN 20 FIELDS IN WOOD COUNTY, OHIO

Table 2 gives the results of the calculations for each field of the 1932 survey of Wood County, Ohio, and serves to illustrate the process carried out for each field in the other counties of the 1931 and 1932 surveys. As an intermediate step in making the substitutions in equation 2, column 9, a, of table 2 gives the value of the term of equation 2 for $n'_b=1$, column 9, b, gives the value of the term for $n'_p=1$, while column 9, c, gives the variance of their product. The values in columns 9, a, and 9, b, may now be divided by the values of $n'_b$ and $n'_p$, respectively, representing the actual number of samples taken in the respective fields, to obtain the values given in columns 10, a, and 10, b. Column 10, c, the sum of columns 10, a, and 10, b, gives the variance of a product when $\sigma^2/n'_b$ and $\sigma^2/n'_p$ are the variances of the means $\overline{b}$ and $\overline{p}$.

TABLE 2.—*Data on European corn borer infestation in 20 fields in Wood County, Ohio, in 1932, illustrating the details of the calculations for obtaining the sampling variance of the mean number of borers per 100 plants in each field*

| Field no. | Statistical constants for number of infested stalks out of 100 stalks | | | Statistical constants for number of borers per infested stalk | | | | The variance of a product when $\sigma_b^2$ and $\sigma_p^2$ are the variances of a single observation $(\overline{p}^2\sigma_b^2+\overline{b}^2\sigma_p^2=\sigma_x^2)$ | | | The variance of a product when $\sigma^2/n'_b$ and $\sigma^2/n'_p$ are the variances of the means $\overline{b}$ and $\overline{p}$ $(\overline{p}^2\sigma^2/n'_b+\overline{b}^2\sigma^2/n'_p=\sigma_{\overline{x}}^2)$ | | | Standard error of product $(\sigma_{\overline{x}})$ | Field mean number of borers per 100 plants |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean infested stalks $(\overline{p})$ | Standard deviation $(\sigma_p)$ | Standard error of mean $\left(\sqrt{\frac{\sigma_p^2}{4}}\right)$ | Mean borers per stalk $(\overline{b})$ | Size of sample $(n'_b)$ | Standard deviation $(\sigma_b)$ | Standard error of mean $\left(\sqrt{\frac{\sigma_b^2}{n'_b}}\right)$ | (9) | | | (10) | | | | |
| | | | | | | | | (a) | (b) | (c) | (a) | (b) | (c) | | |
| (1) | (2) | (3) | (4) | (5) | (6) | (7) | (8) | | | | | | | (11) | (12) |
| 1 | 15 | 3.8 | 1.9 | 1.3 | 10 | 0.82 | 0.26 | 151 | + 24 | = 175 | 15 | + 6 | = 21 | 4.6 | 19.5 |
| 2 | 15 | 7.3 | 3.6 | 1.7 | 10 | 1.42 | .45 | 454 | + 154 | = 608 | 45 | + 39 | = 84 | 9.2 | 25.5 |
| 3 | 33 | 15.1 | 7.5 | 1.7 | 10 | 1.34 | .42 | 1,955 | + 659 | = 2,614 | 196 | + 165 | = 361 | 19.0 | 56.1 |
| 4 | 8 | 10.8 | 5.4 | 1.1 | 8 | .84 | .30 | 46 | + 141 | = 187 | 6 | + 35 | = 41 | 6.4 | 8.8 |
| 5 | 33 | 24.7 | 12.3 | 2.1 | 10 | 1.73 | .55 | 3,259 | + 2,690 | = 5,949 | 326 | + 673 | = 999 | 31.6 | 69.3 |
| 6 | 38 | 15.1 | 7.5 | 2.4 | 10 | 1.43 | .45 | 2,953 | + 1,313 | = 4,266 | 295 | + 328 | = 623 | 25.0 | 91.2 |
| 7 | 46 | 23.7 | 11.8 | 1.3 | 10 | 1.34 | .42 | 3,799 | + 949 | = 4,748 | 380 | + 237 | = 617 | 24.8 | 59.8 |
| 8 | 60 | 11.3 | 5.6 | 1.4 | 10 | .84 | .27 | 2,540 | + 250 | = 2,790 | 254 | + 63 | = 317 | 17.8 | 84.0 |
| 9 | 6 | 9.5 | 4.7 | 1.5 | 6 | 1.38 | .56 | 69 | + 203 | = 272 | 12 | + 51 | = 63 | 7.9 | 9.0 |
| 10 | 12 | 7.8 | 3.9 | 1.2 | 10 | .79 | .25 | 90 | + 88 | = 178 | 9 | + 22 | = 31 | 5.6 | 14.4 |
| 11 | 4 | 3.3 | 1.6 | 1.5 | 4 | 1.00 | .50 | 16 | + 25 | = 41 | 4 | + 6 | = 10 | 3.2 | 6.0 |
| 12 | 6 | 2.3 | 1.1 | 1.8 | 6 | .98 | .40 | 35 | + 17 | = 52 | 6 | + 4 | = 10 | 3.2 | 10.8 |
| 13 | 21 | 8.9 | 4.4 | 2.5 | 10 | 2.17 | .69 | 2,077 | + 495 | = 2,572 | 208 | + 124 | = 332 | 18.2 | 52.5 |
| 14 | 47 | 11.0 | 5.5 | 2.8 | 10 | 1.62 | .51 | 5,797 | + 949 | = 6,746 | 580 | + 237 | = 817 | 28.6 | 131.6 |
| 15 | 27 | 15.1 | 7.5 | 2.1 | 10 | 1.20 | .38 | 1,050 | + 1,006 | = 2,056 | 105 | + 252 | = 357 | 18.9 | 56.7 |
| 16 | 49 | 17.4 | 8.7 | 2.8 | 10 | 2.15 | .68 | 11,099 | + 2,377 | = 13,476 | 1,110 | + 594 | = 1,704 | 41.3 | 137.2 |
| 17 | 80 | 9.8 | 4.9 | 2.1 | 10 | 1.45 | .46 | 13,456 | + 424 | = 13,880 | 1,346 | + 106 | = 1,452 | 38.1 | 168.0 |
| 18 | 37 | 8.9 | 4.4 | 1.6 | 10 | 1.08 | .34 | 1,597 | + 203 | = 1,800 | 160 | + 51 | = 211 | 14.5 | 59.2 |
| 19 | 79 | 6.8 | 3.4 | 1.5 | 10 | 1.35 | .43 | 11,374 | + 104 | = 11,478 | 1,137 | + 26 | = 1,163 | 34.1 | 118.5 |
| 20 | 56 | 31.5 | 15.7 | 2.7 | 10 | 1.57 | .50 | 7,730 | + 7,234 | = 14,964 | 773 | + 1,809 | = 2,582 | 50.8 | 151.2 |
| Sum | | | | | | | | 69,547 | +19,305 | =88,852 | 6,967 | +4,828 | =11,795 | | 1,329.3 |
| Mean | | | | | | | | 3,477.35 | + 965.25 | = 4,442.60 | | | 589.75 | | 66.47 |

SAMPLING VARIABILITY WITHIN ALL FIELDS OF THE SURVEY, GROUPED ACCORDING TO LEVEL OF BORER POPULATION

In order to generalize as completely as possible the information from the entire survey, the fields of all counties were grouped according to the ranges shown in table 3 and by adding and averaging the field means and the variances for $n'_b=1$ and $n'_p=1$ of each term and the totals for the variance of a product. The ranges used in this generalization are entirely arbitrary, table 3 giving a final summary of the variances by field level as finally used. (Fields with means of 4.9 borers per 100 plants or lower are omitted.) In this table is condensed all the information the data afford as to the average magnitude and relationships of the parts of the variance at the different levels of field populations. Each field, representing its own peculiar set of conditions, has its proportionate effect on the final results. The result is not universal but is applicable insofar as these data constitute a representative sample of a given set of conditions encountered in these surveys.

TABLE 3.—*Summary of the sampling variance within fields, grouped within certain ranges of the estimates of the mean number of European corn borers per 100 plants, in the counties of the surveys of 1931 and 1932 in the 1-generation area*

| Group no. | Borers per 100 plants (range) | Fields | Borers per 100 plants (group mean) | Variance of a single observation $(\bar{p}^2\sigma_b^2+\bar{b}^2\sigma_p^2=\sigma_z^2)$ (5) | | | Standard deviation ($\sigma_z$) | Coefficient of variation |
|---|---|---|---|---|---|---|---|---|
| (1) | (2) | (3) | (4) | (a) | (b) | (c) | (6) | (7) |
| | | *Number* | *Number* | | | | *Number* | *Percent* |
| 1 | 5.0- 8.9 | 154 | 6.7 | 26.8 | + 61.7 | = 88.5 | 9.4 | 140.3 |
| 2 | 9.0- 14.9 | 165 | 11.7 | 72.1 | + 121.2 | = 193.3 | 13.9 | 118.8 |
| 3 | 15.0- 24.9 | 145 | 19.5 | 195 | + 210 | = 405 | 20.1 | 103.1 |
| 4 | 25.0- 34.9 | 112 | 29.6 | 425 | + 321 | = 746 | 27.3 | 92.2 |
| 5 | 35.0- 44.9 | 78 | 40.2 | 759 | + 499 | = 1,258 | 35.5 | 88.3 |
| 6 | 45.0- 54.9 | 52 | 49.8 | 1,341 | + 670 | = 2,011 | 44.8 | 90.0 |
| 7 | 55.0- 74.9 | 100 | 65.0 | 2,180 | + 989 | = 3,169 | 56.3 | 86.6 |
| 8 | 75.0- 94.9 | 57 | 83.9 | 3,528 | +1,129 | = 4,657 | 68.2 | 81.3 |
| 9 | 95.0-124.9 | 80 | 109.0 | 5,289 | +1,637 | = 6,926 | 83.2 | 76.3 |
| 10 | 125.0-154.9 | 31 | 137.7 | 8,535 | +2,521 | = 11,056 | 105.1 | 76.3 |
| 11 | 155.0-194.9 | 32 | 171.0 | 13,974 | +2,373 | = 16,347 | 127.9 | 74.8 |
| 12 | 195.0-234.9 | 19 | 211.4 | 21,775 | +3,947 | = 25,722 | 160.4 | 75.9 |
| 13 | 235.0-274.9 | 7 | 254.7 | 31,047 | +3,329 | = 34,376 | 185.4 | 72.8 |
| 14 | 275.0-324.9 | 8 | 295.7 | 48,968 | +2,747 | = 51,715 | 227.4 | 76.9 |
| 15 | 325.0-374.9 | 3 | 354.2 | 47,357 | +2,577 | = 49,934 | 223.5 | 63.1 |
| 16 | 375.0-490.9 | 6 | 423.0 | 73,920 | +2,560 | = 76,480 | 276.6 | 65.4 |
| 17 | 500.0-699.9 | 3 | 525.9 | 80,167 | +3,115 | = 83,282 | 288.6 | 54.9 |
| 18 | 700.0 up | 2 | 970.0 | 180,222 | + 0 | = 180,222 | 424.5 | 43.8 |

Table 3 may be inspected to determine the general characteristics of the sampling variability within the fields. In column 6 are given the generalized standard deviations of the products corresponding to their variances in column 5, c. Column 7 contains the standard deviations expressed as percentages of the generalized means of column 4. These relative values of the standard deviations are statistical constants known as coefficients of variation; they serve as indexes of variability and measure the fluctuation within the fields at the different levels of borer population.

It will be noted that the standard deviations increase in actual magnitude as the means increase, but decrease steadily in relative magnitude from about 40 percent larger than the mean at the lowest

level to less than 50 percent of the mean at the highest level. The part of the variance of the product represented by the term $\overline{b}^2\sigma_p^2$ in column 5, b, of table 3 increases until the mean population reaches 211.4 borers per 100 plants. It then decreases, becoming zero at the highest level. The variance of the infestation samples, the $\sigma_p^2$ part of this term, decreases as the limit of 100-percent infestation is approached. As the square of the mean number of borers per infested plant, the $\overline{b}^2$ part of the term, increases it tends to increase the value of the term, but not at a rate sufficiently great to offset the reduction of the value of the variance of the infestation samples as the limit of 100 percent infestation is approached. At 100-percent infestation the variance of the infestation samples becomes zero and the term vanishes completely.

The part of the variance of the product represented by the term $\overline{p}^2\sigma_b^2$ in column 5, a, of table 3 is the dominant influence in determining the total variance above a level of about 25 borers per 100 plants. This term increases from 57.0 percent of the total variance at a level of about 30 borers to about 96 percent of the total at a level between 400 and 500 borers per 100 plants. Over the range of the populations covered in these data the variability in the number of borers in the infested plants, the $\sigma_b^2$ part of this term, continues to increase as the mean number of borers per infested plant, $\overline{b}$, increases. The square of the number of infested plants per 100, the $\overline{p}^2$ factor of this term, increases up to 10,000 as $\overline{p}$ increases to its maximum of 100. However, increases in population beyond 100 percent infestation must be as a result of increased number of borers per infested plant, which means that any increase in this term is wholly dependent on the magnitude of the $\sigma_b^2$ factor, while the $\overline{p}^2$ factor remains constant.

### Relative Values of Different Combinations of $n'_b$ and $n'_p$

Because of this change in the relative as well as the actual variation at different levels of infestation, it is necessary to study the relative values of different combinations of $n'_b$ and $n'_p$ in sampling at each population level. Three population levels, 11.7, 109.0, and 423 borers per 100 plants were selected from table 3 for study. By substituting in equation 2 the corresponding values from columns 5, a, and 5, b, table 3, and also different combinations of values of $n'_b$ and $n'_p$, the variances of a product for the various sampling combinations for each of the selected levels may be computed. The variances of a product were calculated for all combinations of $n'_b$ and $n'_p$ equal to 1, 2, 3, 4, 5, 7, and 10 for the three selected levels. The standard deviations were then expressed as percentages of the respective means, i. e., as coefficients of variation. The results are shown in the graphs of figure 1.

As noted previously, although the actual magnitude of the standard deviation of the mean for any sampling combination increased as the mean level of population increased, the relative magnitude decreased. Thus, the surfaces of the three graphs of the standard deviations in terms of percent of the mean are increasingly lower as the means increase.

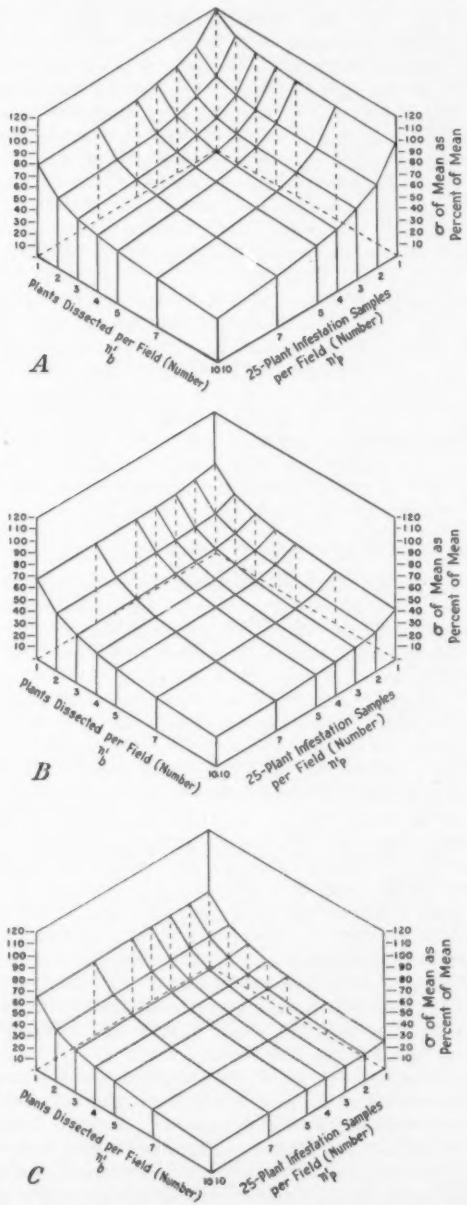


FIGURE 1.—Standard deviations of the field means as percentages of means for different combinations of sampling within fields when field means approximate: *A*, 11.7 borers per 100 plants; *B*, 109.0 borers per 100 plants; *C*, 423.0 borers per 100 plants.

At the level of 11.7 borers per 100 plants (fig. 1, *A*), the second term of the equation (column 5, b, table 3), which is reduced as the number of 25 plant-infestation samples, $n'_p$, is increased, is larger than the first term (column 5, a, table 3), which is reduced as the number of plants dissected, $n'_b$, is increased, so that the total variance is affected more by a comparable increase in the number of infestation samples taken than in the number of plants dissected. At this level the taking of two infestation samples instead of one sample reduces the total variance almost as much as if seven plants had been dissected instead of one plant. However, the predominant effect of taking additional infestation samples on reducing the total variance is soon lost, for at the level of 19.5 borers the taking of two infestation samples instead of one sample reduces the total variance only slightly more than if two plants had been dissected instead of one plant. At the level of 109.0 borers per 100 plants (fig. 1, *B*) the taking of two infestation samples instead of one sample reduces the total variance only about a third as much as if two plants had been dissected instead of one plant, while at the level of 423.0 borers per 100 plants (fig. 1, *C*) the effect is about one-thirtieth as much. At the level of 423.0 borers per 100 plants, increasing the number of infestation samples above two has practically no effect whatever in reducing the sampling variability of the estimate of the mean in such fields.

### OTHER CONSIDERATIONS OF SAMPLING WITHIN FIELDS

In any survey numerous restrictions are always placed on the plan of sampling, solely for eliminating bias rather than because of purely quantitative aspects of the sampling variability. In sampling within the fields in the present survey one such restriction concerned the infestation sample of 25 consecutive plants. Smaller total numbers of plants will suffice for attaining a given degree of reliability in the estimation of the mean percentage of infestation if smaller numbers of consecutive plants are taken at each place selected at random and examined within a field. For example, compared with taking a total of 100 plants, each plant at a place selected at random, it was found that to attain a given reliability in the estimation of the mean percentage of infestation, 171 plants were required when 5 consecutive plants were taken in a place, 206 when 10 plants were taken, 285 when 25 plants were taken, and 660 plants when 100 plants were taken. These results were obtained as an average for five fields with percentages of infestation averaging 38.6, no field having been infested more than 50 percent. Taking the foregoing numbers of plants in the designated manner gave a standard error of 4.71 to the mean of 38.6 percent, but the same proportions would hold among the total numbers of plants required to attain any other degree of reliability in the estimation of the mean of these five fields.

To take each plant of the total number examined for infestation in the fields of this survey in a place selected at random, however, would have necessitated a scheme to eliminate all possible conscious or unconscious bias in taking the plants. Any such scheme would probably be difficult to devise as well as impractical on account of the time necessarily consumed in putting it into effect. For this reason samples of 25 consecutive plants taken in each quarter of the field were arbitrarily chosen from which to determine the percentage of infestation, in the belief that samples of this size would render bias negligible.

The question of the randomness of these samples is essentially the same as that involved in the technique of determining the yield of plots or fields of small grains by harvesting small areas within them. Clapham (*1, 2, 3*) and Kalamkar (*6*) studied such problems and presented experimental data, and on the basis of the analysis of the variance deemed the placement of the samples at definite intervals in the same drill rows to be undesirable. Rather, they recommended that the area be divided into a definite number of equal-sized areas and the samples taken at random in these. The objections noted by these authors to definite placement of samples hardly apply to the manner of taking the infestation samples at approximately the center of the quarters of the fields. These samples were taken without regard to the condition of the corn at the place of sampling in the fields and as an average of a large number of fields the variability would approximate closely to that of fully randomized samples within the quarters of the fields which correspond to sections as recommended by the foregoing authors.

Methods of avoiding bias when taking the infested plants for dissection to determine the number of borers per infested stalk also had to be considered. For this reason only infested plants were dissected in the infestation samples, up to the maximum number specified for the fields as stated previously. It is obvious that in the field the most heavily infested plants would be the ones most easily found, and unless careful safeguards were taken in the methods of the survey to obtain representative plants from the whole field a considerable bias would be introduced.

The most important question in sampling within the fields, however, is the relative weight that should be given to the samples of the two sets of factors and this question is answered by the solutions carried out on the basis of the equation for the variance of products. As used also, the actual sampling variability within the fields, while important in properly planning area-wide surveys, is distinctly subordinate to the field-to-field variability within the larger areas, in its final effect on the reliability of the estimate of the county mean number of borers per 100 plants.

## ANALYSIS OF THE FIELD-TO-FIELD VARIABILITY WITHIN COUNTIES

### METHODS OF ANALYSIS OF VARIANCE APPLIED TO SAMPLING VARIABILITY OF AREA-WIDE SURVEYS

The foregoing analysis treated of the sampling variability within the fields so that estimates could be obtained of the true levels of infestation of each field, these in turn being used to obtain estimates of the mean levels of infestation of the counties. Within the counties the actual level of infestation will vary greatly from field to field. The differences between the fields constitute another component of the variability affecting the reliability of the estimates of the mean populations of the county areas, and this component is of paramount importance in the specification of the methods of sampling to be used in the surveys. To measure and study the characteristics of this component of the variability by itself, and in comparison with the components of the sampling variability within the fields, actual observations under the conditions to be studied must be used. The data for the field estimates of the mean number of borers per 100 plants

for the 66 counties already used for the study of the sampling variability within the fields constitute the data used for this study of the field-to-field variability within counties.

The fields sampled were selected at predetermined points distributed fairly uniformly over the county, thus insuring, to the greatest possible degree, both representativeness and randomness in the samples. The first step is to determine by the methods of the analysis of variance devised by Fisher (*4*) whether the fields within sections of the counties tended to be more nearly alike in level of infestation than the fields over the whole counties—in other words, to see whether there were significant differences in level of infestation between the sections of the counties. For this study each county was divided into four sections of approximately equal area but more or less arbitrarily delimited so as to have each section include the same number of fields in as compact units as possible. The allocation had to be done after the surveys were carried out, but care was exercised to avoid any consideration of the values in making up the sections, these being based entirely on location. In counties in which 20 fields were sampled each section included 5 fields and in counties in which 25 fields were sampled each section included 6 fields. (Fields numbered 13 in the counties of 25 fields were uniformly and arbitrarily discarded and county means and other values adjusted accordingly.) The solutions were carried out by the methods of the analysis of variance to determine (1) the total variation of the field means from the county mean, (2) the variation of the field means within the sections from the means of their respective sections, and (3) the variability attributable to the differences between the mean population levels in the four sections. These parts were later related to the sampling variability within fields, solved in the preceding section of the paper.

## DATA FOR SURVEY OF WOOD COUNTY, OHIO, IN 1932 USED TO ILLUSTRATE THE SOLUTION

The values for the mean numbers of borers per 100 plants for the 20 fields surveyed in Wood County in 1932 will be used to illustrate the solutions which were applied to all counties. These values are given in column 12 of table 2 and the manner of grouping the fields into the four sections is shown in figure 2; while the values, arranged according to sections for the analysis of the variance, are given in table 4.

TABLE 4.—*Mean number of European corn borers per 100 plants by fields for the survey of Wood County, Ohio, in 1932, arranged in sections for the analysis of the variance*

| Section 1 | | Section 2 | | Section 3 | | Section 4 | |
|---|---|---|---|---|---|---|---|
| Field no. | Mean number borers | Field no. | Mean number borers | Field no. | Mean number borers | Field no. | Mean number borers |
| 1 | 19.5 | 3 | 56.1 | 10 | 14.4 | 12 | 10.8 |
| 2 | 25.5 | 4 | 8.8 | 15 | 56.7 | 13 | 52.5 |
| 5 | 69.3 | 8 | 84.0 | 16 | 137.2 | 14 | 131.6 |
| 6 | 91.2 | 9 | 9.0 | 17 | 168.0 | 19 | 118.5 |
| 7 | 59.8 | 11 | 6.0 | 18 | 59.2 | 20 | 151.2 |
| Total | 265.3 | | 163.9 | | 435.5 | | 464.6 |

Examples of the application of the methods of the analysis of variance to similar problems and detailed discussions of the concepts involved are given in numerous publications including Fisher (*4*), Wishart (*9*), and Snedecor (*8*). Although all these vary somewhat in the particular arithmetic procedure, the basic concepts are the same.

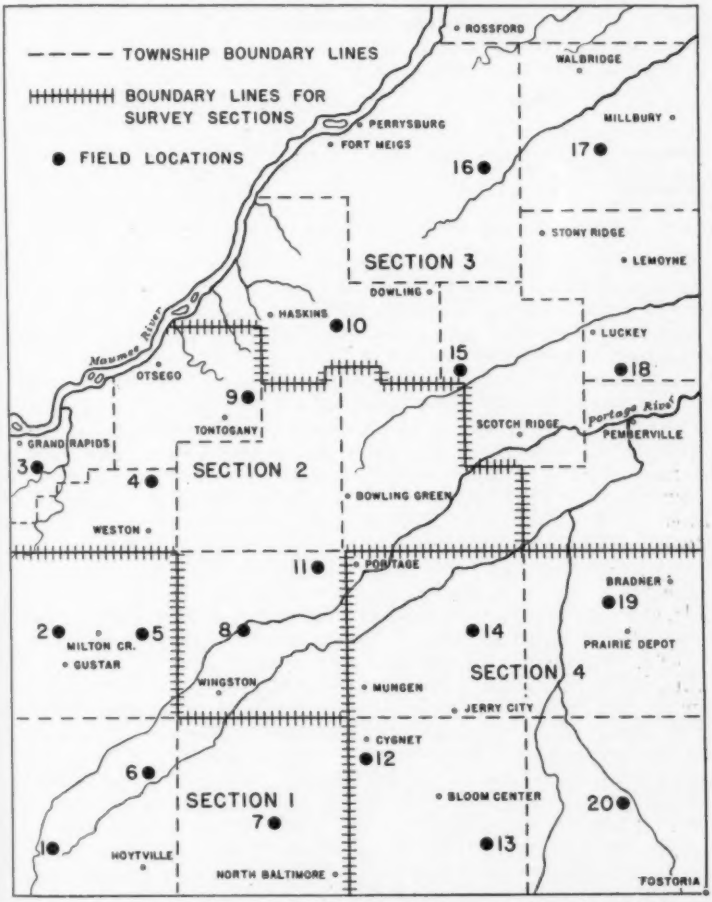


FIGURE 2.—Map of Wood County, Ohio, showing the location of the fields sampled in 1932 in the survey of the number of borers per 100 plants, and the allotment into four sections for the analysis of the field-to-field variability within the county.

Specifically, this particular problem is identical with the class given by Snedecor (*8*) as example 1, page 3, of his manual. This type of problem has a single criterion of classification and an equal number of observations in each of the classes.

From table 5 it will be noted that the sum of the squares of the deviations of the 20 field means from the section means repre-

senting 16 degrees of freedom is less than the sum of the squares of the deviations of 20 field means from the county mean representing 19 degrees of freedom. The difference is attributable to the 3 degrees of freedom representing the deviations of the population levels of the four sections from the general county mean.

TABLE 5.—*Analysis of the variance in the mean number of European corn borers per 100 plants in Wood County, Ohio, in 1932*

| Variation | Degrees of freedom | Sum of squares | Mean-square deviation | Standard or root-mean-square deviation |
|---|---|---|---|---|
| Total for county | 19 | 50,842.07 | 2,675.90 | 51.73 |
| Within sections | 16 | 38,641.85 | 2,415.12 | 49.14 |
| Between sections | 3 | 12,200.22 | 4,066.74 | 63.77 |

The values in the last two columns of this solution for the mean-square deviation (variance) and the root-mean-square deviation are directly comparable in magnitude within the columns.

Assuming that real differences were indicated between the actual population levels of the sections as laid out in Wood County in 1932, the mean-square deviation or variance between sections in table 5 may be considered as being made up of two parts, the first with variance of $A_1$, representing the estimated actual differences in level of population between sections, and the second or $B_1$ part, which is the mean of the variance within sections for the individual fields shown above. This phase of the problem has been discussed by Fisher (*4*) in section 40. The $A_1$ part is found from the difference between 4,066.74 and 2,415.12, or 1,651.62, while the $B_1$ part is the mean variance between fields within sections, or 2,415.12. The values of the mean-square deviation in table 5 are the variances of single fields, and since a section comprises 5 fields, the $A_1$ part of the variance of the section mean number of borers per 100 plants is one-fifth of 1,651.62, or 330.32. The square root of this, 18.17 borers per 100 plants, is the estimated standard error of the real levels of population from section to section, corrected for the sampling variability within the sections. This discussion is more readily understood by assuming that the mean-square deviation between sections approximates the mean-square deviation within sections so closely that the difference between them is well within the limits set for errors of random sampling. Then the variability between fields in one section and fields in other sections would not be significantly greater on the average than the variability from field to field within the sections and the $A_1$ part of the variance would not exist.

But the $A_1$ part of the variability within counties is irrelevant to the reliability of the estimates of the county means if at least one field is sampled in each section, and it should be eliminated in the estimate of the true sampling variability. In replicated field experiments Fisher (*5*) terms this method "local control", but it can be applied as well to methods of area-wide insect surveys. It is evident, therefore, that only the $B_1$ part, or the variance of the field means within the sections, applies to the reliability of the estimates of the county

means. In the solution as carried out here the local control is applied by dividing the county into four sections. The 20 or 25 fields sampled in these surveys were distributed over the counties, but were arbitrarily grouped into 4 sections as being the solution combining most advantages in the study. In order to carry out any formal solution for the estimate of the parts of the variance, there must be at least two assumed sections and at least two fields in each section. If there are the same number of sections in each county the results may be combined for study later. Also, in the study hereinafter carried out, if there are four sections per county the estimated variability of county means will proceed by four-field intervals, which is a convenient unit.

Just as the total variability of the field means from the county mean was separated into two parts, so also may the $B_1$ part of the variance which applies to the reliability of sampling in the county be divided into two parts for more detailed study of the sampling methods. The first part, designated as the $A_2$ part, represents the estimated variance of the real levels of population of the fields within the sections, and the second part, designated as the $B_2$ part, represents the variability of the sampling within the fields for the estimation of the field means and will be taken at $n'_p = n'_b = 1$. This $B_2$ part is obtained directly from column 9, c, in table 2. The total 88,852 is divided by the number of fields (20) sampled in the county, which gives a mean-square deviation of 4,442.6. The square root of this value, 66.65 borers per 100 plants, is the corresponding standard deviation. The components of this 4,442.6, affected respectively by modifications in the numbers of plants dissected to determine the number of borers per infested plant and in the numbers of samples taken to determine the percent infestation, are obtained in the same manner from the corresponding columns, i. e., 9, a, and 9, b, of the same table. The mean-square deviation affected by the number of plants dissected is 3,477.35 and that affected by the number of infestation samples is 965.25, designated as the $C_2$ and $D_2$ parts of the variance, respectively.

The $A_2$ part is equated from the $B_1$ part as the $A_1$ part was equated from the variance of the section means. The $B_1$ part, or the variability of the field means within the sections, is made up of a part, designated as the $A_2$ part, which represents the difference of the true field levels from the section levels, and a part due to the variability of the sampling within the fields. The latter is estimated from column 10, c, of table 2. This column gives the estimated total variance of sampling within each field for the actual sampling combination used. Each field has to be figured separately owing to the differences in the number of plants dissected in the various fields, otherwise this step could be figured in one operation, as in subtracting the $B_1$ part from the variance between sections for estimating the $A_1$ part of the variance. The sum of this column, 11,795, divided by 20 gives the average variance within the fields, or 589.75. This is subtracted from the variance within sections, $B_1$, or 2,415.12, giving as $A_2$ 1,825.37, or the estimated variance of the true levels of population of the fields from the section levels; that is, it gives the estimated variance if all field levels and section levels were known without error. The square root of this value, 42.72 borers per 100 plants, is the corresponding standard deviation among the fields within sections.

The sampling within the fields, however, contributes to the variability of the fields within the counties and thus influences the reliability of the county means obtained as estimates of the true population levels. The $B_2$ given above for $n'_p=n'_b=1$, and its parts, $C_2$ and $D_2$, are taken for the basic estimate of these values for counties for the purpose of standardization. The sum of $B_2$ and $A_2$ is the estimated total variance of fields within sections when 1 sample of 25 consecutive plants is examined to determine the percentage of infestation, and 1 infested plant is dissected to determine the number of borers per infested plant in each field. The square root of this value, 79.17 borers per 100 plants, is the corresponding total standard deviation for fields within sections.

This completes the analysis of the variability of the samples as actually taken in the county. The information that has been derived is summarized in table 6.

TABLE 6.—*Summary of analysis of the variance in surveys of European corn borer populations, Wood County, Ohio, 1932*

| Estimated variability of— | Variance | | Standard deviation, borers per 100 plants |
|---|---|---|---|
| | Designation | Value | |
| | | | *Number* |
| Section levels from county level | $A_1$ | 330.32 | 18.17 |
| Fields levels from section level | $A_2$ | 1,825.37 | 42.72 |
| Sampling within fields ($n'_p=n'_b=1$) | $B_2$ | 4,442.60 | 66.65 |
| Total of estimates of field means ($n'_p=n'_b=1$) from section means | $A_1+B_2$ | 6,267.97 | 79.17 |

SAMPLING VARIABILITY FOR THE COUNTIES GROUPED BY LEVEL OF POPULATION

An analysis similar to that made in Wood County, Ohio, was made for each of the other 65 counties of the 1931 and 1932 surveys in the one-generation area in which the sampling variability within fields was studied. The counties were then arranged according to the mean numbers of borers per 100 plants, and the variances added and averaged over specified ranges as shown in table 7. The ranges used here are entirely arbitrary, as in the case of the ranges used for the study of the variability of the sampling within fields. Corresponding standard deviations, both as actual values and as percentages of the group means of the numbers of borers per 100 plants, are also given in the table.

TABLE 7.—*Summary of the sampling variance within counties of the estimates of the mean number of European corn borers per 100 plants for the counties of the 1931 and 1932 fall surveys of infestation of the one-generation area studied*

| Group no. | Borers per 100 plants (range) | Counties | Borers per 100 plants (group mean) | Mean variances for— | | | | Standard deviations for— | | | | | | | | Variances affected by— | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Section levels from county levels ($A_1$) | Field levels from section levels ($A_2$) | Sampling within fields ($B_2$) | Total ($A_2+B_2$) | Section levels from county levels | | Field levels from section levels | | Sampling within fields | | Total | | Number of plants dissected ($C_2$) | Number of infestation samples ($D_2$) |
| | | | | | | | | $\sqrt{A_1}$ | As percent of mean | $\sqrt{A_2}$ | As percent of mean | $\sqrt{B_2}$ | As percent of mean | $\sqrt{A_2+B_2}$ | As percent of mean | | |
| | *Number* | *Number* | *Number* | | | | | *Number* | *Percent* | *Number* | *Percent* | *Number* | *Percent* | *Number* | *Percent* | | |
| 1 | 1– 4.99 | 6 | 2.68 | 0.49 | 3.7 | 30.5 | 34.2 | 0.70 | 26 | 1.92 | 72 | 5.52 | 206 | 5.85 | 218 | 8.3 | 22.2 |
| 2 | 5– 9.99 | 6 | 8.88 | 11.1 | 56.0 | 184.2 | 240.2 | 3.33 | 38 | 7.48 | 84 | 13.57 | 153 | 15.50 | 175 | 86.2 | 98.0 |
| 3 | 10– 19.99 | 12 | 13.60 | 23.6 | 139.0 | 366.5 | 505.5 | 4.86 | 36 | 11.79 | 87 | 19.14 | 141 | 22.48 | 165 | 193.2 | 173.3 |
| 4 | 20– 29.99 | 8 | 24.71 | 62.9 | 603.1 | 943.5 | 1,546.6 | 7.93 | 32 | 24.56 | 99 | 30.72 | 124 | 39.33 | 159 | 649.1 | 294.4 |
| 5 | 30– 44.99 | 6 | 36.59 | 39.0 | 905.6 | 1,871.5 | 2,777.1 | 6.24 | 17 | 30.09 | 82 | 43.26 | 118 | 52.70 | 144 | 1,367.8 | 503.7 |
| 6 | 45– 64.99 | 7 | 57.55 | 730.0 | 2,734.2 | 3,965.7 | 6,699.9 | 27.02 | 47 | 52.29 | 91 | 62.97 | 109 | 81.85 | 142 | 3,032.4 | 933.3 |
| 7 | 65– 79.99 | 13 | 70.75 | 578.7 | 4,439.9 | 5,632.7 | 10,072.6 | 24.06 | 34 | 66.63 | 94 | 75.05 | 106 | 100.36 | 142 | 4,742.2 | 890.5 |
| 8 | 80–119.99 | 5 | 99.61 | 1,688.2 | 9,153.4 | 10,197.4 | 19,350.8 | 41.09 | 41 | 95.67 | 96 | 100.98 | 101 | 139.11 | 140 | 8,934.2 | 1,263.2 |
| 9 | 120 and up | 3 | 158.74 | (1) | 32,394.6 | 20,371.6 | 52,766.2 | (1) | ------ | 179.98 | 113 | 142.73 | 90 | 229.71 | 145 | 18,482.3 | 1,889.3 |

[1] None.

Examination of the results in table 7 reveals that the total standard deviation relevant to the sampling of the county means, $\sqrt{A_2+B_2}$, increases in actual magnitude as the mean level of population increases in the counties. This standard deviation also decreases in relative magnitude but with no pronounced trend at the higher levels of borer population. The standard deviation of the $A_1$ part of the variance, which is the estimated variance of the real section levels from the county levels of population and is eliminated by the principle of local control, and therefore is not relevant to the reliability of the estimates of the county means, increases in actual magnitude as the mean level of population increases in the counties. The percentage this standard deviation bears to the county means is almost constant. The standard deviation of the $A_2$ part of the variance, which is the estimated variance of the real field-to-field differences within sections, increases in both actual and relative magnitude as the county levels of population increase.

The standard deviation of the $B_2$ (for $n'_p=n'_b=1$) part of the variance, which is the variance of sampling within the fields, increases in actual magnitude and decreases in relative magnitude as the county levels increase. Within the fields the parts of the $B_2$ variance $C_2$ (which is affected by the number of plants dissected, $n'_b$) and $D_2$ (which is affected by the number of infestation samples, $n'_p$) show somewhat the same characteristics on the basis of counties as they did for the individual fields, when classified by population levels. Both parts increase in actual magnitude as the county levels increase, but the $C_2$ part increases very much more rapidly than the $D_2$ part.

These characteristics of the parts of the variability of sampling within the counties all influence the relative effectiveness of different methods of sampling in estimating the levels of populations in various areas. Figure 3 shows the standard deviations of county means for various sampling combinations for the three levels 8.88, 57.55, and 99.61 borers per 100 plants taken from table 7.

The values for figure 3 were derived as follows: Let $n'$ equal the number of sections per county (in this analysis assumed to be 4) and let $k$ equal the number of fields sampled per section. Then the variance of the estimates of the county means for any sampling combination is given by the equation

$$\sigma^2 \text{ county mean} = \frac{A_2+\frac{C_2}{n'_b}+\frac{D_2}{n'_p}}{n'k}. \qquad \text{Equation 5}$$

As the number of fields sampled in a county is increased over four the area is divided into an equal number of sections of uniform size and one sample taken in each to accomplish the greatest possible amount of local control, thus shifting more of the $A_2$ part of the variance to the $A_1$ part, but it was not practical with these data to carry out a solution which conformed strictly to this principle. Finally, the standard errors of the means for the various sampling combinations are stated as percentages of the corresponding means. Corresponding values for all sampling combinations for $n'=4$ with $k$ values from 1 to 10 (4 to 40 fields) and $n'_b=1$ and 5, and $n'_p=1$ and 4 were computed for the three levels shown in figure 3.

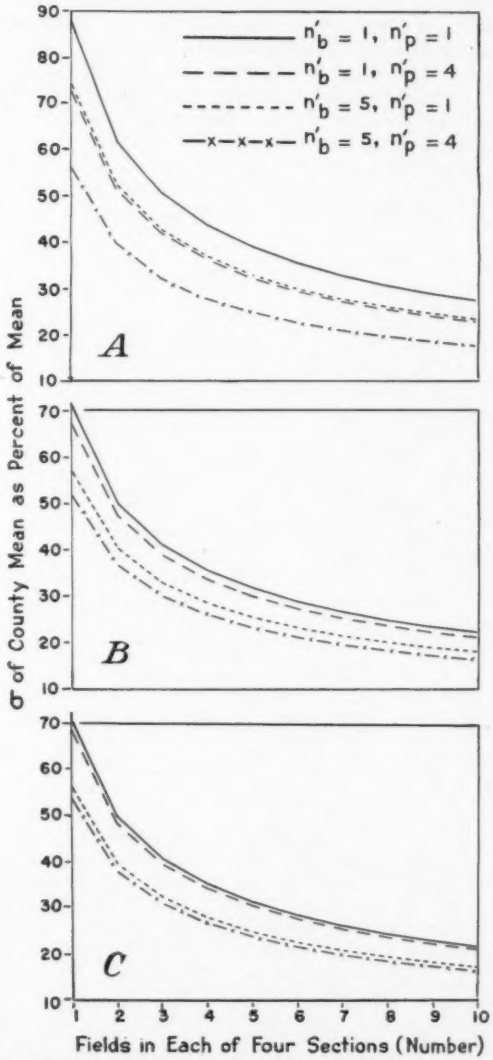


FIGURE 3.—Standard deviations of county means as percentages of the county means for various combinations of samples within the fields and various numbers of fields sampled within the county for means of: *A*, 8.88 European corn borers per 100 plants; *B*, 57.55 borers per 100 plants; *C*, 99.61 borers per 100 plants.

At the level of 8.88 borers per 100 plants, increasing the number of plants dissected, $n'_b$, from 1 to 5 within each field has nearly the same effect in reducing the standard deviation of the estimates of the county means as increasing the number of 25-plant infestation samples, $n'_p$, from 1 to 4, and increasing both $n'_b$ and $n'_p$ by these amounts at the same time is about twice as effective. As the levels of population of the counties increase, the reduction in the variability of the county means due to increasing the number of infestation samples decreases rapidly, and the curve for $n'_b=1$ and $n'_p=4$ approaches the curve for $n'_b=1$ and $n'_p=1$, and the curve for $n'_b=5$ and $n'_p=1$ approaches the curve for $n'_b=5$ and $n'_p=4$. The characteristics of the various parts of the variability of sampling in this survey are thus revealed.

## DISCUSSION

In the past, several studies have been conducted relative to the methods of sampling to be followed within fields in surveys of insect abundance. From the standpoint of the methods of conducting area-wide surveys those studies give information only on a relatively less important part of the total variance of sampling, whereas they really should apply to the estimates of the mean of the larger areas. Furthermore, the fields in which the sampling studies were conducted often were selected and limited in number, thus still further limiting the applicability of the results. It is only from actual survey data, such as are used in this paper, that estimates of all the essential parts of the sampling variability can be obtained. This information on the characteristics of the sampling variability in the surveys is obtained along with the current collection of information on the status of the insect population. The specifications which effectively meet these two requirements in a survey are practically the same.

The method of sampling at the beginning of a survey must be based on such information as may be at hand, including the knowledge of the biology of the insect and host, matters of agronomy, the general principles of survey sampling, the objects of the survey, similarity to other surveys that have been conducted, and the reconnaissance observations that have been carried out previously on the same problem. It should not be difficult to arrive at specifications fairly near the most efficient ones possible by making use of all these factors. As the data accumulate in the survey they are analyzed in reasonable detail and the desirable improvements in methods inaugurated.

The three basic principles of survey sampling which are essential to meet the requirements of obtaining the most reliable estimate of the area means, and the proper measurement of the parts of the sampling variable, are: (1) Replication or multiple samples in each subdivision of the survey (i. e., counties or fields); (2) local control, or dividing the area to be sampled (counties or fields) into as many sections, uniform in size or representing aliquot parts of the host organism to be sampled, as there are samples to be taken; and (3) randomness, or taking one of the specified samples at random in each section.

Replication increases the reliability of the estimates of the area means, such as counties or fields, directly in proportion to the number of samples. The larger the number of samples taken, therefore, the greater the reliability of the estimate of the mean, although as the number becomes larger the relative effectiveness of this factor per unit sample becomes less and less. When combined with the principle of local control, however, the effectiveness of the replication is usually greatly enhanced.

The sampling variability which applies to the reliability of the estimates of the means is the variability within sections for each phase of sampling (county or fields in this survey). As the number of sections is increased, more and more of the total variability of the given phase is shifted from the part of the variability from sample to sample within the sections, that which is relevant to the variability of the estimates of the means (*5*), to the part of the variance for differences between the levels of the sections, that which is irrelevant to the variability of the estimates of the means. Thus, by the combination of the principles of multiple samples and local control, the variability of the estimates of the corresponding means is reduced much more rapidly than the square root of the number of samples operating on the total variability of the unit samples. If for any reason the samples are grouped in clusters, some of the advantage of local control is lost and the solution for the analysis of the variance must be modified accordingly. This is true even though it is necessary to consider two or more of the samples of a given area of the survey together in a section of correspondingly larger size for the analysis of the variance. Some of the true section-to-section variability which is irrelevant to the variability of the estimates of the means is of necessity included within sections because of this limitation in the solution for actual survey data, but the entire gain due to local control by placing all samples in uniform sections actually is inherent in the estimate of the mean itself. The discrepancy usually is not very great. Randomness in taking the samples within the section is, of course, absolutely essential for obtaining either an unbiased estimate of the corresponding means or a correct estimate of the sampling variability.

Although these principles of sampling apply equally to sampling within fields or between the fields of a county, the phases differ greatly in relative effectiveness, per unit sample, on the reliability of the estimates of the means. Increasing the number of samples of the phases involving successively larger unit areas has much more effect on the reliability of the final means than like increases in the successively smaller units, although this advantage is offset to some extent by the greater cost of increasing the number of samples in the larger areas. The mathematical relationship in this survey may be obtained from equation 5. The increased effectiveness of additional samples on the reliability of the means becomes less pronounced as the number of samples increases, owing to the fact that the reliability of estimates of a mean is directly in proportion to the number of samples. No one phase, therefore, is increased indefinitely without regard to the others, and a proper balance in sampling is maintained.

## SUMMARY

The data used for the analysis of the sampling variability in this study consisted of the records of the 1931 and 1932 fall surveys, for the number of European corn borers per 100 plants, of a total of 66 county units located in the States of Indiana, Michigan, Ohio, Pennsylvania, and New York, infested by the one-generation strain. The methods of determining the sampling variability within the fields, for the estimation of the field means, and the variability from field to field within the county (part of which pertains to the reliability of the estimates of the mean level of population in the counties) were analyzed in considerable detail.

In sampling within the fields 4 units of 25 consecutive plants were first examined to determine the number of infested plants out of 100 plants; then from 5 to 10 of these infested plants were dissected to furnish an estimate of the average number of borers per infested plant. The product of these two means gave the estimates of the field mean number of borers per 100 plants. The equation for the estimation of the variance of products from the variance of the factors was used to obtain the estimates of the variance of these field mean numbers of borers per 100 plants. This method expressed the variability of sampling within the fields in terms that permitted the direct comparison of the contributions of any combination of the two sets of factors to the final variability of the estimate of the field mean.

The variability from field to field within the counties was divided into three main portions by the methods of the analysis of variance. The first portion consisted of the variability between county sections each of which was arbitrarily chosen to include one-fourth of the fields sampled in the county. This portion of the variability, which represented the estimated actual differences in level between the sections, is irrelevant to the estimate of the reliability of the county means, and illustrates the effect of applying the principle of local control in sampling in area-wide surveys of insect abundance.

The variability of field means from the respective section means is divided into two portions, one representing the estimates of the variability of the true field levels from the respective section levels, and the other representing the variability of the estimates of the field means for a given system of sampling within the fields.

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